

DARK SEPTATE AND ARBUSCULAR MYCORRHIZAL FUNGAL ENDOPHYTES
IN ROOTS OF PRAIRIE GRASSES

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By

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ABSTRACT

Root symbioses with dark septate endophytic fungi (DSE) and arbuscular mycorrhizal fungi (AMF) provide plant tolerance to environmental stresses. This research answers several fundamental questions about the occurrence of these fungi in roots of prairie grasses. Traditional methods and current molecular techniques were combined in order to: 1) define the role and specificity of DSE in plant tolerance to drought; 2) assess the level of host specificity in DSE; 3) document AMF biodiversity and pattern of root colonization at different soil depths; 4) define the influence of soil depth and plant species on the distribution of DSE and AMF in roots and; 5) reveal how DSE and AMF interact in plant roots.

Under controlled conditions, DSE isolates showed host preference in colonizing roots and promoting plant growth. They colonized with more intensity the plant species from which they were isolated [*Agropyron cristatum* L. or *Psathyrostachys juncea* (Fisch) Nevski subsp. *Juncea* (Syn: *Elymus junceus* Fisch)]. Inoculation with five DSE isolates resulted in growth stimulation of the C₃ grasses *A. cristatum* and *P. juncea*, and growth depression of the C₄ grass *Bouteloua gracillis* (Willd. ex Kunth) Lag. ex Griffiths, under water stress. Plant C concentration suggested that DSE inoculation may have resulted in net C drain from *B. gracillis*.

In the field, soil depth influenced root colonization in *A. cristatum*, *Panicum virgatum* L., *Nassella viridula* Trin and *Pascopyrum smithii* (Rydb.) A. Löve., while AMF diversity was influenced by the interaction between soil depth and host plant species. Molecular analysis of roots serially sampled during one growing season from the A and B soil horizons, in stands of these grasses, revealed spatial and temporal changes in DSE and AMF community composition, and a significant correlation in DSE and AMF community structure.

These results suggest that DSE and AMF are adapted to specific environmental conditions and that root occupation by these fungi is a dynamic phenomenon. It is proposed that temporal

variation in root occupation by DSE and AMF impacts plant and ecosystem processes at different times during the growing season.

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LIST OF ABBREVIATIONS

AC: *Agropyron cristatum*

AM: Arbuscular mycorrhiza

AMF: Arbuscular mycorrhizal fungi

AMMI: Additive main effect and multiplicative interaction

ANOSIM: Analysis of similarities

ANOVA: analysis of variance

BG: *Bouteloua gracillis*

bp: base pairs

DGGE: Denaturing gradient gel electrophoresis

DNA: Deoxyribonucleic acid

DSE: dark septate endophyte (or endophytic) fungi

GINCO: Glomales international collection

Glo: *Glomus*

GTR + G: General time reversible with gamma substitution model

INVAM: International culture collection of arbuscular and vesicular-arbuscular mycorrhizal
fungi

IPCA1: Interaction principal component axis 1

LSU: Long subunit

MANOVA: Multivariate analysis of variance

MDS: Multidimensional scaling

MPa: mega Pascal

NSERC Canada: Natural Sciences and Engineering Research Council of Canada

NV: *Nassella viridula*

PCR: Polymerase chain reaction

PDA: Potato dextrose agar

PJ: *Psathyrostachys juncea*

PS: *Pascopyron smithii*

PV: *Panicum virgatum*

rDNA: Ribosomal DNA

SIMPER: Similarity percentage

SPARC: Semiarid Prairie Agricultural Research Centre

SSU: Short subunit

TRFLP: Terminal restriction fragment length polymorphism

TRF(s): Terminal restriction fragment(s)

1 INTRODUCTION

Symbioses with dark septate endophytes (DSE) and arbuscular mycorrhizal fungi (AMF) are common in terrestrial ecosystems, where they provide tolerance to several soil conditions that may restrict plant growth. AMF symbioses are particularly important for plant uptake of slow diffusing soil nutrients (Brundrett, 2004), whereas the role of DSE is associated with increased plant tolerance to extreme temperature (Marquez et al., 2007) and saline (Waller et al., 2005) soil conditions, although DSE and AMF are phylogenetically distant organisms. DSE is a broad term used to describe fungi with melanized, septate hyphae asymptotically colonizing plant roots (Jumpponen, 2001). Members of this group do not belong to a particular phylum, although apparently they can be placed within the Ascomycota and Basidiomycota (Summerbell, 2005; Zijlstra et al., 2005). The AMF are taxonomically related and have similar lifestyle. They collectively form the Glomeromycota (Schüßler et al., 2002).

An increasing number of reports suggest that simultaneous colonization of plant roots by DSE and AMF is common in nature and widespread in plant ecosystems. Despite the co-occurrence of DSE and AMF in plant roots, the study of these symbioses as a whole is scarce, and the current knowledge about DSE and AMF is unequal. While a wealth of literature supports the multiple roles of AMF symbiosis on plant fitness, little is known about the role of DSE symbioses, although microscopic or molecular analyses of roots generally showed that DSE can be more abundant than AMF (Mandyam and Jumpponen, 2008; Santos-Gonzalez et al., 2007; Weishampel and Bedford, 2006)

The simultaneous study of DSE and AMF in symbiosis with plant roots is challenging. They are genetically unrelated and appear to have different levels of host preference or function in different environmental limits. It is apparent that DSE are generalists, since the presence of the

same fungal species had been reported in distant ecosystems colonizing roots of multiple and unrelated host plants (Jumpponen and Trappe, 1998), whereas AMF appear to show higher levels of host plant preference, as suggested by the detection of specific AMF-host plant assemblages in a similar ecosystem (Gollotte et al., 2004).

For feasibility reasons, DSE and AMF are generally separated for their study. However, from the perspective of classical ecological theories and according to current knowledge on the symbiotic behavior of DSE and AMF, these groups should be studied together. DSE and AMF commonly co-occur in plant roots and their endophytic status suggests that both have access to plant synthesized organic compounds (Pearson and Jakobsen, 1993; Usuki and Narisawa, 2007). It is not known if DSE and AMF may compete for plant C compounds, but competing interactions could lead to functional and spatial separation of the two fungal communities into distinct niches (Gadgil and Gadgil, 1971; Lindahl et al., 2007). DSE and AMF symbioses co-occur around the globe and their possible interactions are unknown. Increased knowledge on how these cosmopolitan root symbionts interact will help to understand the mechanisms that maintain root endophyte diversity and stabilize symbiotically mediated plant processes.

Environmental heterogeneity is important to maintain genetic diversity (Koch, 2006). The Canadian prairie ecozone is characterized by a seasonal succession in the activity of plant species and environmental conditions. Good water availability and a landscape dominated by cool season C₃ vegetation early in the season is in contrast to high temperature and prolonged drought periods to which C₄ and late season plants are adapted. Environmental conditions also vary with soil depth. Prairie plants commonly possess a deep root system (Zajicek et al., 1986) which crosses soil layers with different physico-chemical properties. This heterogeneous rooting zone should lead to heterogeneous root endophyte communities. However, knowledge on variation in

composition of root endophytic communities with soil depth or their interactions with plant roots in the Canadian prairie ecozone is lacking.

The relevance of DSE and AMF in plant processes is supported by a wealth of knowledge obtained from the microscopic observation of colonized roots, but the resolution of this approach is limited. The composition of fungal root endophytes is dynamic and changes in space and time. Although changes in the intensity or pattern of root colonization can be detected under the microscope, the changes in community composition may remain overlooked if they are based on hyphal counts. DSE and AMF communities are genetically complex, and at least in AMF, genetic differences between species or isolates of a same species, had been associated with different symbiotic functioning (Maherali and Klironomos, 2007; Munkvold et al., 2004). Studies of complex communities, like those occurring in roots would be greatly benefited from the use of methods with enough resolution to discriminate broad fungal groups and individuals within them.

Molecular and biochemical methods are available to study aspects of fungal community diversity and ecology with a high resolution (Anderson and Cairney, 2004). These methods may help to shed much needed light on the functionality of DSE and AMF symbiosis. In particular, terminal restriction fragment length polymorphism (TRFLP) combined with cloning of DNA is a highly reproducible method which allows identification of DNA from particular organisms in complex mixtures of DNA usually obtained from soil or roots (Campbell et al., 1996; Singh and Thomas, 2006).

In this research traditional microbiological and new molecular methods were implemented in order to answer fundamental questions about the DSE and AMF symbioses, which co-occur in

plant roots. Parallel field and indoor studies with grasses growing in the Canadian prairie ecozone were used in order to define:

- 1 The influence of DSE isolates on grass growth under conditions of reduced water availability and their level of specificity in host root colonization
- 2 The biodiversity and distribution of AMF at different soil depths
- 3 The influence of sampling times and soil depth on the composition of DSE and AMF communities during the growing season, and to understand how these communities interact.

Chapter 2 reviews general aspects about DSE and AMF symbioses in plant roots, which are relevant to this research. The isolation and assessment of specificity in root colonization and grass growth promotion of DSE isolates under reduced water availability, as well as the approximate classification of DSE isolates based on phylogenetic analysis of ribosomal DNA (rDNA) sequences is reported in Chapter 3. In Chapter 4, AMF biodiversity and root colonization at two soil depths were defined using microscopic analysis, and molecular and phylogenetic tools. For Chapter 5, specific molecular markers were designed using DSE fungal cultures and cloned AMF rDNA, in order to track temporal and spatial changes in DSE and AMF communities during the growing season, and to discover potential relationships between the structures of these communities. All results are integrated and globally discussed in Chapter 6. Finally, the appendix presents the experiments designed to select suitable PCR primers which were used in this research for molecular analysis of DSE and AMF.

2 LITERATURE REVIEW

2.1 Divergent Nature of Root Symbiosis with Dark Septate Endophytes (DSE) and Arbuscular Mycorrhizal Fungi (AMF)

Symbioses with dark septate endophytes (DSE) and arbuscular mycorrhizal fungi (AMF) co-occur in roots of many plant species in terrestrial ecosystems. Symbioses with AMF are particularly important for plant uptake of slow diffusing soil nutrients (Brundrett, 2004), while many non mycorrhizal fungal endophytes appear to play an important role in plant adaptation to extreme environmental conditions (Rodriguez et al., 2008; Waller et al., 2005).

Most studies of plant root symbioses have focused on nutritional benefits provided by AMF. However, the ecological role of AMF could go far beyond nutritional effects. In AMF symbioses, extra radical hyphal networks reach soil nutrients that are beyond the reach of roots and transfer these nutrients to plants across internally developed root-hypha interfaces (Brundrett, 2004). Additional benefits from AMF symbiosis may include the reduction in plant diseases (Newsham et al., 1995; St-Arnaud and Vujanovic, 2007), heavy metal absorption (Leyval et al., 1997) and improved soil water relations (Augé, 2001).

Although some members of the AMF are genetically divergent, they are all grouped within the phylum Glomeromycota and are highly dependent on the presence of a plant host for growth (Rosendahl, 2008). Symbioses with AMF may have played an important role during the early land colonization of by primitive plants, as suggested by the presence AMF like structures in fossil records (Remy et al., 1994). Today the AMF symbiosis appears to play an important role in plant community dynamics, as inferred from the fact that plant roots are connected to common underground mycorrhizal networks that might distribute nutrients among neighboring plant species (Giovannetti et al., 2004). Despite the cosmopolitan distribution of certain AMF species

(Koch et al., 2004), some degree of host specificity (Gollotte et al., 2004) and environmental adaptation also appear to exist in AMF (Appoloni et al., 2008).

While a wealth of research provides support to mycorrhizal fungi as enhancers of plant nutrition and stress tolerance, limited evidence suggests that DSE may perform a similar role in dry environments (Barrow, 2003). DSE is a broad term which describes a group of taxonomically heterogeneous fungal species with melanized, septate hyphae that asymptotically colonize plant roots (Jumpponen and Trappe, 1998).

The symbiosis with fungal root endophytes does not always benefit plants (Kageyama et al., 2008), and fungi lifestyles can vary from mutualism, in one environment, to parasitism in another (Bethlenfalvay et al., 1983; Schulz and Boyle, 2005). While the mutualistic lifestyle provides benefits to the plant, the parasitic life style is detrimental.

Root colonization by DSE can be more abundant than colonization by arbuscular mycorrhizal fungi (AMF) in the roots of prairie grasses (Porrás-Alfaro et al., 2008). Despite their ubiquitous presence, the ecological role of DSE is unclear. In *Bouteloua* species, root colonization by DSE has been associated with drought tolerance (Barrow et al., 2004). The roots colonized by DSE showed a mucilaginous polysaccharide layer that was presumed to protect plants against desiccation and keep hydraulic root soil continuity under dry conditions (Barrow et al., 2004). However, *Bouteloua* species are naturally adapted to drought through their C₄ metabolism (Moore et al., 2004). It is not known if the physiological response to DSE inoculation observed in *Bouteloua* also occurs in plants more sensitive to drought, for instance C₃ plant species, which have shown improved drought tolerance in symbiosis with other fungal root endophytes (Rodríguez et al., 2008).

Although some DSE can contribute to plant nutrition (Mullen and Schmidt, 1993) and adaptation to soil extreme conditions (Marquez et al., 2007), the mechanisms are not well understood, and the costs associated with this symbiosis are not known. Some authors argue that the absence of specific cytological features during root-DSE interactions, which are normally developed with biotrophic fungi, shed doubt on the occurrence of nutrient exchange between DSE and plants (Peterson et al., 2008). Furthermore, as opposed to AMF, DSE can grow as saprotrophs in the absence of a host plant.

Many fungi that can be broadly classified as a DSE are taxonomically unrelated and belong to separate phyla. Many DSE, as well several fungi involved in other specialized types of mycorrhiza improving plant growth (Summerbell, 2005) or N nutrition (Usuki and Narisawa, 2007; Zijlstra et al., 2005), belong to the Ascomycota.

2.2 Co-Occurrence of Dark Septate Endophytes and Arbuscular Mycorrhizal Fungi in Plant Roots

Recent reports suggest that co-occurrence of DSE and AMF in plant roots is common in nature. Co-occurrence of DSE and AMF was reported in medicinal and aromatic plants in southern India (Muthukumar et al., 2006), grasslands across Europe (Santos-Gonzalez et al., 2007), North America (Mandyam and Jumpponen, 2008; Porras-Alfaro et al., 2008) and Asia (Lingfei et al., 2005), in high altitude habitats in Asia and America (Li and Guan, 2007; Schmidt et al., 2008), in alpine environments in Finland and North America (Mullen and Schmidt, 1993; Ruotsalainen and Kytöviita, 2004), in montane forest in Costa Rica (Rains et al., 2003) in wetlands in the US (Weishampel and Bedford, 2006) and in plants of the Cholistan Desert in Pakistan (Chaudhry et al., 2005).

Despite the frequent co-occurring DSE and AMF in plant roots, the current knowledge about these two groups is unequal, which greatly limits the comprehension of these symbioses as a

whole. Analyses of databases in the ISI web of knowledge®, conducted on June 11 of 2009 illustrates this situation. A search using the strings “arbuscular mycorrhiza OR amf”; “dark septate OR radicis atrovirens OR dse” or both strings combined in the “TOPIC” search option, found 3464, 1080 and 40 records since 1900.

Few studies based on microscopic analysis focused on DSE and AMF together. However, from the perspective of classical ecological theories, since DSE and AMF co-occur in roots and may have access to plant synthesized organic compounds, these fungi should be studied as a whole. It is not known if DSE and AMF may compete for plant supplied C, but if so, their competing interactions could lead to functional and spatial separation of these fungal groups, as suggested before (Gadgil and Gadgil, 1971; Lindahl et al., 2007). DSE and AMF symbioses co-occur around the globe, and knowledge about their ecological interactions is scarce.

Microscopic observations indicated that DSE and AMF may have different seasonal dynamics of root colonization (Mandyam and Jumpponen, 2008), or different distribution in soil horizons (Medina-Roldan et al., 2008). DSE also may be more abundant in roots than AMF in grassland ecosystems (Medina-Roldan et al., 2008). This is important since each symbiosis might provide tolerance to stressful conditions or help plants to use resources occurring at different times during the year (Schmidt et al., 2007). Microscopic based studies have limitations and should be complemented with more discriminating approaches. DSE (Sieber and Grünig, 2005) and AMF (Sanders, 2004a) show a remarkable genetic diversity, but from microscopic observations it is not possible to know if differences in dynamics of root colonization by DSE and AMF communities is the result of shifts in relative abundance of specific individuals, or the entire DSE or AMF community.

2.3 The Challenge of Studying DSE and AMF Symbioses as a Whole

The large genetic diversity along with the divergent nature of DSE and AMF makes it difficult to study them together with enough resolution to differentiate the contributions of particular groups or individuals to root colonization. Individual genetic differences in fungal root endophytes are relevant since they can be related to differences in symbiotic functioning (Koch et al., 2006; Munkvold et al., 2004), adaptation to different hosts (Gollotte et al., 2004) or perhaps overall environmental conditions.

2.4 Dynamics of Root Niches For DSE and AMF in the Prairie Environment

From a fungal endophyte perspective, niches in prairie grass roots are ever changing targets. The prairie ecozone is characterized by a seasonal succession in vegetation and environmental conditions. Low temperature and good water availability support the activity of cool season C_3 vegetation early in the season, which contrasts with the high temperature and prolonged drought periods during which C_4 and drought adapted plants may remain active, in the second part of the growing season. Variability in environment also occurs due to soil depth, because prairie grass roots can cross heterogeneous soil layers (Craine et al., 2003), which exposes its endophytes to multiple environmental conditions. AMF can show preferential association with roots of specific plant species (Gollotte et al., 2004) and specific DSE or AMF genotypes have been detected in specific portions of the roots (Sieber and Grünig, 2005), or at specific soil depths (Oehl et al., 2005). However, there are no clues about the factors governing the distribution of DSE and AMF in plant roots. Root endophytes can be affected by surrounding soil conditions (Dodd et al., 2000) and it seems possible that distinct niches are available in roots at different soil depths or at different times during the growing season. This is important since different fungi might contribute differently to plant fitness by promoting the use of resources differentially distributed along the soil profile at different times. This structured distribution of niches may lead to a

microbially mediated niche separation, which is a key mechanism to sustain plant and fungal diversity in ecosystems (Reynolds et al., 2003).

2.5 Importance of Biodiverse DSE and AMF Communities

Levels of AMF diversity or genetic variability were previously related to plant productivity or ecosystem stability (van der Heijden et al., 1988). For instance, intra species variants may show high variability in mycelia length and improvement in plant P nutrition, with low impact on plant growth (Munkvold et al., 2004), while a number of distantly related AMF species are more important in providing stability to plant productivity than the same number of closely related species (Maherali and Klironomos, 2007). As opposed to AMF, differences in symbiotic behavior of DSE at the subspecies level are not known. However, siderophore production, a function which might greatly impact plant nutrition, is differentially affected by pH in isolates of the same DSE species (Bartholdy et al., 2001).

Most of the knowledge on the diversity and functions of AMF has been gained by identifying spores and observing stained roots obtained from the top 10 or 15 cm soil layer. This approach which provided fundamental knowledge about the importance and ecology of AMF in soil plant ecosystems has limitations. It has low resolution power and can overlook AMF taxa with sparse or periodic spore production (Rosendahl, 2008) or that might be adapted to deeper soil layers (Oehl et al., 2005). In addition, different isolates of the same species may function or respond differently to environmental changes (Sanders, 2004b), although these different isolates may produce identical spores. Given the complex genetic and phenotypic organization of DSE and AMF communities in plant roots, it is desirable to complement microscopic observations with additional tools. Specifically, molecular methods have high discriminating power and can be used to track and identify DSE and AMF under varied experimental conditions (Redecker, 2000; Rosendahl, 2008).

2.6 PCR Based Analysis of Microbial Communities

Several molecular methods are available to study fungal community diversity and ecology. Specific genes can be used to fingerprint species or isolates or to aid in fungal identification and diversity assessment under natural conditions (Redecker, 2000; Rosendahl, 2008). In particular, terminal restriction fragment length polymorphism (TRFLP) is used to study microbial communities and its variations across environmental gradients. Compared to other community analysis methods, TRFLP has higher resolution power and provides reproducible results (Singh and Thomas, 2006). One disadvantage is that particular microbial species cannot be identified after a DNA fragment is analyzed, and comparison between communities is based only on the diversity of length variable, unidentified terminal DNA fragments generated by restriction enzymes. However, the combination of TRFLP analysis and cloning of DNA allows tracking microbial species in natural environments. In this approach, reference terminal restriction fragments (TRFs) are generated either from computer simulated restriction digestion of DNA sequences, or from the digestion of the same cloned DNA which generated the sequence. The length of these reference TRFs is then compared to the length of TRFs produced from PCR amplified and restriction enzyme digested DNA for detection in samples of interest (Campbell et al., 2004).

Combined TRFLP and cloning is a molecular tool suitable for high throughput analysis of microbial biodiversity, although this combination involves a highly skilled labour. Once the method is chosen, the selection of primer sets for PCR amplification of DNA is probably the most critical step to ensure success and efficient use of resources.

2.7 Choice of Primer Sets for PCR Based Analysis of Fungal Root Biodiversity

Primer pairs selection is probably the most critical step when biodiversity analysis is based on PCR amplified DNA. Numerous primers for AMF or for fungi in general are published

(Anderson and Cairney, 2004; Clapp et al., 1995; Gollotte et al., 2004; Hagn et al., 2003; Redecker, 2000; Renker et al., 2006; Sato et al., 2005; Simon et al., 1992; Trouvelot et al., 1999; Van Tuinen et al., 1998).

Good specificity and good range of amplification are two desirable properties of PCR primers. Ideal primers amplify only the DNA from members of the target group of organisms and from all members of that particular group. The specificity and range of amplification by PCR primers is usually taken for granted, or in a better situation it is obtained from DNA sequence analysis in public data bases, although these tests are rarely reported or included as an integral part of a molecular biodiversity project.

The risk of amplifying non target DNA sequences is high in PCR based analysis of biodiversity when the target DNA is part of a complex mixture of DNA templates, like that found in soil or plant roots. For example, DNA of *Phialophora* was recovered from DGGE separated bands of DNA amplified with the AMF primers AM1 and NS31 (Ma et al., 2005). Clearly these primers are not AMF-specific and may lead to erroneous conclusions if the amplified sequences are not duly identified. Insuring the specificity of the primer pairs used is important in biodiversity studies where conclusions are drawn from unidentified tagged DNA fragments or bands, such as those produced by terminal restriction fragment length polymorphism (TRFLP) or denaturing gradient gel electrophoresis (DGGE), which are two popular environmental DNA analysis methods (Kirk et al., 2004).

In PCR based biodiversity assessments, the primers might be tested in advance. Specificity and range of PCR primers can be tested detecting priming sites in DNA sequences available in public databases, or by direct PCR amplification of DNA from samples of interest (Hagn et al., 2003). The first approach is easier, but these methods can yield very different results. Published

primers intended to amplify DNA only from fungi, can show a wide range of amplification, with good specificity towards DNA from fungi in sequence databases. However, the same primers showed unspecific and/or narrow range of amplification of DNA extracted from reference samples which included fungal cultures (Hagn et al., 2003). In vitro tests appear to be the safest way to select suitable primers sets for PCR based biodiversity assessments.

3 GRASS GROWTH PROMOTION UNDER WATER STRESS AND ROOT COLONIZATION BY DARK SEPTATE ENDOPHYTIC FUNGI IS HOST SPECIFIC

3.1 Abstract

Little is known on the role of dark septate endophytic fungi (DSE) in the prairie ecozone, despite their abundance in the roots of grasses. For this research, five isolates of ascomycetous DSE were obtained from roots of crested wheatgrass (*Agropyron cristatum* L.), Russian wildrye [*Psathyrostachys juncea* (Fisch) Nevski subsp. *juncea* (Syn: *Elymus junceus* Fisch)] and blue gramma grass [*Bouteloua gracillis* (Willd. ex Kunth) Lag. ex Griffiths]. The response of these grass species to inoculation with each of the DSE isolates was assessed in a factorial experiment conducted under controlled conditions and water stress. The capacity of two isolates obtained from *A. cristatum* and *P. juncea* to colonize the roots of the grass species from which they were isolated was also tested in sterile microcosms.

Plant growth response and root colonization by DSE depended on the plant host. It was generally positive in the water stressed cool season grasses *A. cristatum* and *P. juncea*, and negative in *B. gracillis*. The DSE, which apparently were a C drain on plant photosynthesis, always decreased the shoot C concentration of *B. gracillis*. One fungal isolate increased leaf N concentration, as compared to the control. In sterile microcosms each isolate colonized more intensely the roots of the grass species from which they were isolated.

In conclusion, root colonization and growth promotion by DSE may be more specific than previously thought. It appears from these results that DSE may regulate the growth and perhaps distribution of the grass species included in this research, mainly through drought stress reduction in *A. cristatum* and *P. juncea* and parasitism in *B. gracillis*.

3.2 Introduction

The symbiosis between endophytic fungi and plant roots is prevalent in nature. These symbioses usually provide fitness to plant and fungi and involve virtually any known plant with fungi that forms mycorrhiza or with dark septate endophytic fungi (DSE). While symbioses with mycorrhizal fungi are well studied little is known about symbioses with DSE. The DSE are a group of taxonomically heterogeneous species with melanized hyphae colonizing the roots of plant species asymptotically that form symbiosis with plants in different ecosystems (Jumpponen and Trappe, 1998)

Symbioses with fungal root endophytes does not always benefit the plant (Kageyama et al., 2008), as they can vary from mutualism, in one environment, to parasitism in another (Schulz and Boyle, 2005). Therefore, the same fungal isolate can have contrasting effects on two different hosts (Kageyama et al., 2008) or in the same host under different soil conditions (Bethlenfalvay et al., 1983), and in roots of prairie grasses growing southern in the United States, DSE can be more abundant than arbuscular mycorrhizal fungi (AMF) (Porrás-Alfaro et al., 2008). While a wealth of research provides support to mycorrhizal fungi as enhancers of plant nutrition and stress tolerance, limited evidence suggests that DSE may perform a similar role in dry environments (Barrow, 2003). Despite their ubiquitous presence in roots of plants growing in the Canadian prairie ecozone, the ecological role of DSE is unclear. In particular their plant growth promotion capacity and level of host specificity are unknown.

The Canadian prairie environment is characterized by marked seasonal variations. Cooler temperatures and good soil water availability typically prevail in early summer and warmer temperatures and drought conditions are usual in late summer. A clear shift in vegetation occurs as the season progresses, with ‘cool season plants’ dominating in early season, i.e. in May and

June, and ‘warm season plants’ becoming competitive during the hot and dry months of July and August.

Fungal isolates were obtained from the roots of three common grass species growing in the Canadian prairie ecozone and used to test the hypothesis that DSE improve plant growth under conditions of low water availability. It was also verified under axenic conditions that some DSE can have host preference for root colonization. This research presents evidence of specificity in DSE related mitigation of drought stress in plant, and host preference in DSE isolates.

3.3 Materials and Methods

3.3.1 Isolation of dark septate endophytes

Five fungal isolates were obtained from the roots of field grown *A. cristatum* and *P. juncea* or *B. gracillis* grown indoor in natural soil. The soil or plant stands used for this research were located at the South farm of the Semiarid Prairie Agricultural Research Centre, SK. Canada, (latitude 50° 18' N; longitude 107° 41' W).

Root pieces three to five centimeters in length were surface sterilized by successive immersion in 95% ethanol for 10 s, sterile water for 10 s, 2.5% Javex® for 2 min, and sterile water for 2 min. Sterilized roots were then cut into pieces 0.3- to 0.5-cm long using a sterile scalpel, plated on PDA supplemented with neomycin sulphate (12 mg L⁻¹) and streptomycin sulphate (100 mg L⁻¹) (Vujanovic et al., 2002), and incubated in the dark at 25°C. After four to 60 d, hyphae emerging from the cut end of root fragments were transferred to new Petri plates to obtain pure cultures. Pieces of PDA colonized by the fungi were stored in a 0.8% NaCl solution (Dhingra and Sinclair, 1995) at 4° C until further use. The isolates obtained and referred to hereafter are: AC1, BG17, PJ2, AC4, and PJ5. The first two letters in the code used for naming DSE isolates refer to the host plant from which the fungus was isolated, i.e., AC = *A. cristatum*, PJ = *P. juncea*, BG = *B. gracillis*.

3.3.2 Test of plant growth promotion by DSE isolates

The capacity of fungal isolates to promote plant growth was tested using mesocosms in a complete randomized factorial experiment with five replicates. Germinated seeds of the grasses *A. cristatum*, *P. juncea* and *B. gracillis* were inoculated with the DSE isolates AC1, BG17, PJ2, AC4, PJ5 or sterile PDA plugs.

The mesocosms consisted of plastic pots containing 450 g of pasteurized soil (90° C, 1 h) with the soil water content adjusted to 150 g kg⁻¹ of soil. Five inoculated seedlings were transplanted into each mesocosm. Each pot received 10 mL of water daily during the first week, then the plants were thinned to two per pot and 25 mL of water was added every morning until the end of the experiment. This quantity of water equals 55 g kg⁻¹ of soil. Soil chemical analysis was: NH₄-N 19.72 mg kg⁻¹, NO₃-N 14.13 mg kg⁻¹, K 357 mg kg⁻¹, PO₄-P 21.92 mg kg⁻¹, Organic C 5.7 g kg⁻¹ and total N 0.8 g kg⁻¹. The mesocosms were kept under 23:19°C and 16:8 hours light: dark cycles, with relative humidity of 60% and 445 μmol m⁻² s⁻¹ of photosynthetically active radiation, and were re-randomized every second day in the growth chamber. Gross transpiration rate in each mesocosm was estimated gravimetrically on day 25, by subtracting the mass of water lost through evaporation from the mass of water added to each pot 24 h before. Soil water evaporation was estimated as the average of water lost during that period from three randomly placed non-planted pots containing the same quantity of soil. Transpiration rate was expressed as mL of water per gram of dry weight per day. Plant shoots were harvested after 44 days, dried at 55°C, weighed and ground in a bead mill. To assess the potential effect of DSE on the level of drought stress experienced by the C₃ plants *A. cristatum* and *P. juncea* (Farquhar and Richards, 1984), and on source of soil N used by plants, concentration of C and N, and natural abundance of their heavier isotopes δ¹³C and δ¹⁵N were determined on a Carlo Erba

NA1500 elemental CN analyzer coupled to an Optima isotope ratio mass spectrometer. Dry mass and shoot C concentration are presented as relative values, which were calculated as the ratio of values in a plant-fungi combination to averaged values measured on non-inoculated plants of the same species grown under similar conditions.

3.3.3 Test of host preference by DSE isolates

The host preference for colonization by two DSE isolates was tested using sterile microcosms in a factorial experiment with three replicates. In this experiment surface sterile seeds of the grasses *A. cristatum* and *P. juncea* were inoculated with isolates AC1, PJ5 or sterile PDA plugs. Sterile microcosms consisted of borosilicate test tubes 15 cm long and two cm in diameter, filled with an eight cm layer of quartz sand and a top 2.5 cm layer of soil (Scher et al., 1984). Five mL of a 1:1000 diluted 20-20-20 fertilizer were added to each microcosm, which was then sterilized for 1h at 115°C, 0.138 MPa. Sterile plugs of PDA 2 mm in diameter, or plugs from 3 wk old cultures of isolates AC1 or PJ5 were taken with a cork borer and placed over the emerging root of germinated, surface sterile seeds of each grass species. Seed surface sterilization followed the procedure described above for root surface sterilization. Inoculated seeds were kept overnight on wet filter paper in Petri dishes before being transferred into different microcosms. The lower portion of the tube containing substrate and the opening were covered with aluminum foil and placed with a 45° inclination in the growth cabinet. The microcosms were kept under the same growth conditions described above and moved to a randomly selected position in the growth cabinet once a week. Sterile water was added under aseptic conditions as needed. The full root system in each mesocosm was harvested after 75 d and washed over a 100-mesh sieve. Root samples were stained using a 5% solution of Schaeffer black ink in vinegar (Vierheilig et al., 1998) and percent colonization assessed under a compound microscope at 400X magnification using the line intercept method (Giovanetti and Mosse, 1980). Finally all roots in each

microcosm were scanned and measured using the image analysis software WinRHIZO®. The treatments were replicated three times.

3.3.4 Statistical analysis

Two-way Manova was used to estimate the main effect of plant species and fungi inoculation or their interaction on response variables. In the test of plant growth promotion by DSE isolates, values of shoot dry mass, concentration (%) of C and N, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of each plant species were standardized in a 0 to 1 scale (Wilkinson and Engelman, 2007). Manova was followed by analysis of simple effects if a significant interaction ($P < 0.05$) between plant species and DSE isolates was detected. Dunnett T3 tests or custom contrasts were used to test the simple effect of DSE inoculation on relative shoot dry mass or C concentration, respectively. Dunnett T3 tests were used to compare relative dry mass means since their variance was heterogeneous (Levene's test $P \leq 0.05$). The main effect of inoculation on leaf N concentration was tested by Dunnett one side tests (Dunnett and Gent, 1996). In the test of host preference by DSE isolates, values of root colonization were transformed [$\sqrt{(\% \text{colonization} + 1)}$] to meet the requirement of normality before statistical analysis. Manova was followed by contrasts analysis in order to analyze the simple effect of DSE inoculation on host root colonization. Statistical analyses were performed in Systat 12 (SYSTAT software, Inc. Chicago, IL).

3.3.5 Phylogenetic classification of dark septate endophytes

Approximate identification of DSE was based on PCR amplification, cloning and sequencing of the 5' end of the long subunit ribosomal DNA (LSU rDNA). DNA was extracted from a 0.25 cm² piece of 3 wk old mycelia grown in PDA media. The sample was dried at 50°C in micro centrifuge tubes during 24 h and then ground in a bead mill. Two hundred μL of TE buffer and 50 μL of a 20% Chelex 100 suspension in TE buffer were added to each tube, mixed by hand

during 20 seconds and heated at 95°C (5 min). The tubes were then incubated on crushed ice (1 min) and centrifuged at 10000 rpm (1 min). One μL of the supernatant was diluted in 199 μL of TE buffer and immediately used for PCR. Twenty μL of PCR cocktail contained 1 μL of diluted DNA, 18.34 μL of PCR platinum master mix (Invitrogen Corp) and 0.08 μL of a 50 μM solution of primers LR1 (Van Tuinen et al., 1998) and FLR2 (Trouvelot et al., 1999). The amplification included one step of 4 min at 94°C, followed by 35 cycles of 1 min at 93°C, 1 min at 60°C and 1 min at 72°C; and a final step of 10 min at 72°C. PCR products were analyzed by gel electrophoresis and cloned using the Topo TA cloning kit (Invitrogen Corp) according to the manufacturer's instructions. Plasmids PCR amplified with primers LR1-FLR2 and containing a fragment of rDNA 600 bp long were sequenced using primers T3-T7 at the Plant Biotechnology Institute, Saskatoon (NRC Canada). rDNA sequences of DSE showing similarity [$>95\%$ using the Basic Local Alignment Search Tool (Blast search) (Wheeler, 2003) on Jun 11 of 2009] to the rDNA sequences obtained in this research, several sequences of fungal orders that contain root endophytes (Rodriguez et al., 2008; Summerbell, 2005) and sequences reported in the most complete fungal phylogeny published to date (James et al., 2006) were aligned using Clustal W. The phylogenetic analysis was obtained from Bayesian, distance and parsimony approaches. Bayesian analysis in the software MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) used the next settings: generations 1000000, burnin time 35%, model GTR+G and tree sampling each 500 generations. Additional support for the branches was obtained from 1,000 bootstrap iterations in a Neighbor joining three built with Tamura Nei distances (Tamura et al., 2007). Models of nucleotide substitution for both trees were obtained using PhyML (Guindon and Gascuel, 2003). A consensus tree from maximum parsimony analysis was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the

random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 231 positions in the final dataset, out of which 100 were parsimony informative. Confidence of the branches was obtained from 500 bootstrap iterations. Parsimony and Neighbor Joining analyses were conducted in the software MEGA4 (Tamura et al., 2007). Sequences of DSE rDNA obtained in this study were deposited in Genbank under accession numbers EU635769 to EU635773.

3.4 Results

3.4.1 Plant growth promotion by DSE isolates

A DSE x plant species interaction ($P = 0.007$) revealed that growth response to inoculation with DSE isolates AC1, BG17, PJ2 and AC4 was usually negative in *B. gracillis* and positive in *A. cristatum* and *P. juncea* (Fig. 3.1a). Isolate PJ5 did not have significant effect on relative dry mass of the grass species studied (Fig. 3.1a). A DSE x plant species interaction was also found on relative shoot C concentration ($P = 0.006$), which responded similarly to plant growth, generally increasing in inoculated *A. cristatum* and *P. juncea* and decreasing in *B. gracillis* (Fig. 3.1b).

Plant transpiration rate, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were not significantly affected by DSE inoculation or their interaction with plant species. Shoot N concentration was significantly increased ($P = 0.034$) by inoculation with isolate PJ2 only (Fig. 3.2).

3.4.2 Host preference by two DSE fungal isolates

Both fungal isolates, AC1 and PJ5, showed higher colonization in the plant species from which they were isolated (fungal DSE isolate x plant species interaction, $P < 0.01$). Root colonization by isolate AC1 was 28 times higher in *A. cristatum*, the plant species from which this isolate was obtained, than the colonization in *P. juncea*. Similarly, root colonization by PJ5,

which was isolated from roots of *P. juncea* was three times higher in *P. juncea* than in *A. cristatum* (Fig. 3.3). There was no significant effect of DSE inoculation on root length.

3.4.3 Phylogenetic classification of dark septate endophytes

All fungal isolates were Ascomycetes. Isolates BG17, PJ5, AC4 and PJ2 were placed within the order Pleosporales and isolate AC1 was placed within the order Helotiales (Fig. 3.4). All clades containing the DSE fungal endophytes were significantly supported by the phylogenetic analysis. These DSE isolates are related to genera of other endophytic Ascomycetes with capacity to improve plant growth (Marquez et al., 2007; Summerbell, 2005) or N nutrition (Zijlstra et al., 2005) under diverse experimental conditions

3.5 Discussion

Results showed that Ascomycetous DSE associated with grasses commonly found in the prairie ecozone can improve the growth of grass species under drought stress. The growth response and extent of root colonization by these fungi depend on both: the plant and the fungus.

Several studies have reported plant growth stimulation in DSE symbioses, and because DSE can colonize the roots of different hosts, they were traditionally seen as relatively non-specific plant symbionts (Jumpponen and Trappe, 1998). In contrast to common belief, none of the DSE isolates in this research provided benefits to *B. gracillis*, and all but one produced a positive growth response in *A. cristatum* or *P. juncea*, indicating that DSE do not always promote the growth of plants, and that the growth response they cause in this particular water-stressed grasses is largely host specific. The differential effects of DSE on plant growth observed here might be explained at least partially by physiological differences between these grass species. *Bouteloua gracillis* is a C₄ plant (Moore et al., 2004), while *A. cristatum* and *P. juncea* possess a C₃ photosynthetic metabolism. The C₄ metabolism is an adaptation to dry environments that

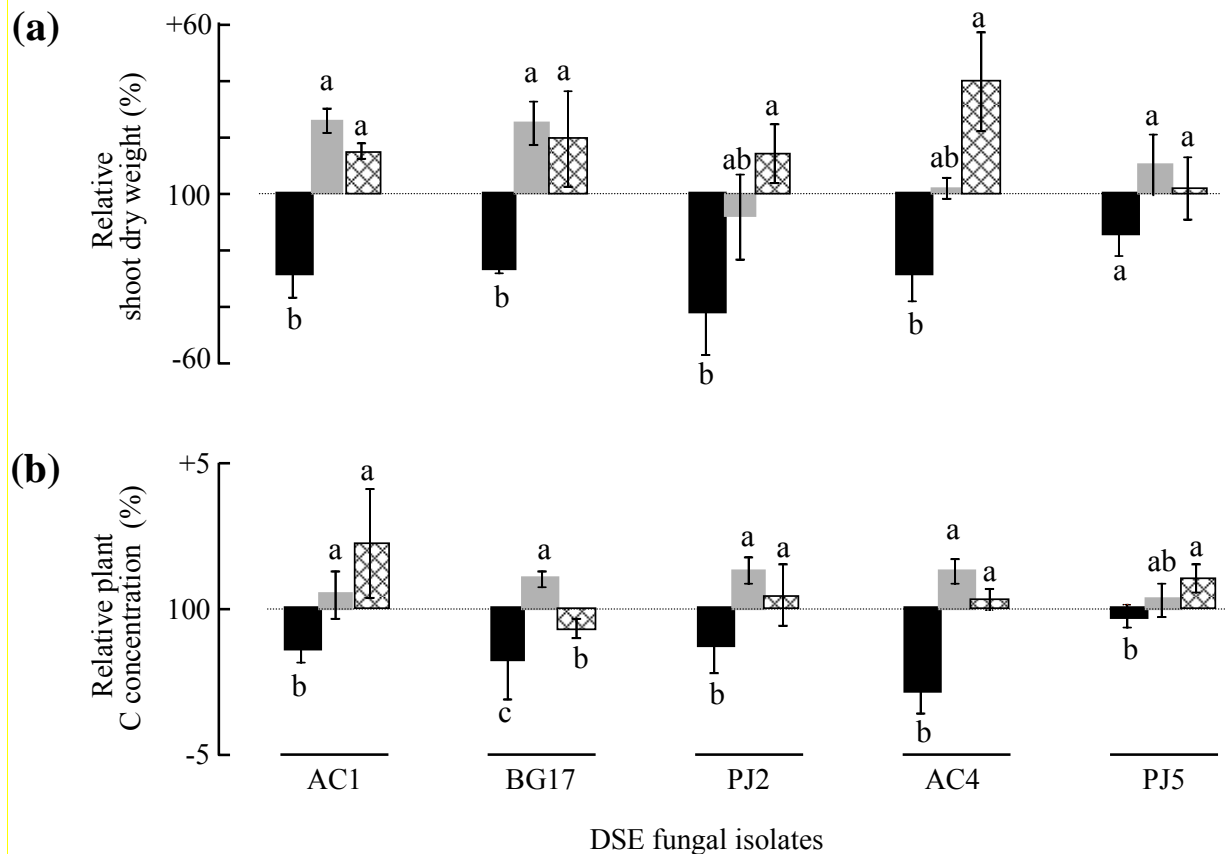


Fig. 3.1 Effect of inoculation of grass species with five DSE isolates on a) plant dry mass and b) plant C concentration. Values are the ratios of inoculated to mock inoculated control plants (100%). Bars represent least square means with one standard error. The dotted line drawn at 100% represents the non-inoculated control plants. Within each DSE isolate, bars with different letters indicate significant ($P < 0.05$) differences in shoot dry mass (Dunnett T3 test, homogeneity of variances not assumed [Levene's P test < 0.05]) or shoot C concentration (custom contrasts) response to inoculation. Solid bars = *B. gracillis*, grey bars = *A. cristatum*, cross stacked bars = *P. juncea*. $n = 5$ in all plant * DSE isolate combinations, except in *B. gracillis* * BG17 ($n = 3$) and *B. gracillis* * PJ2 ($n = 4$).

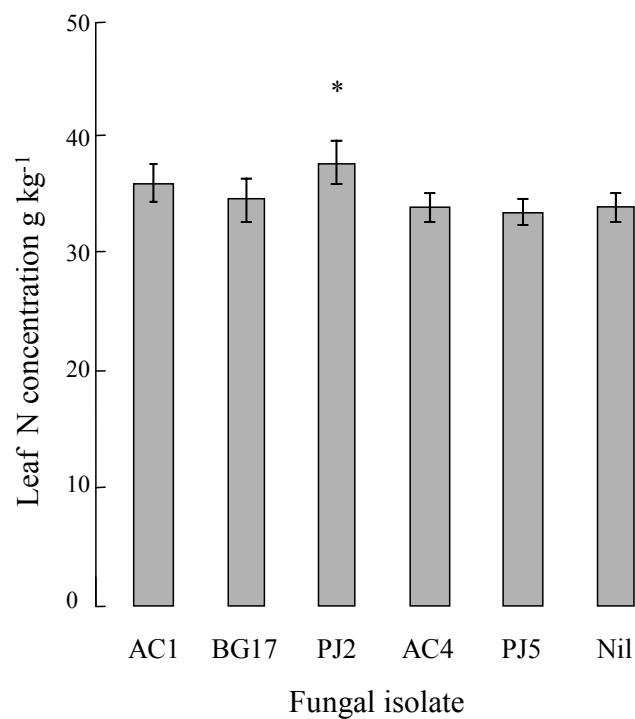


Fig. 3.2 Effect of DSE inoculation on leaf N concentration ($P = 0.034$). Bars represent least square means, with one standard error of values averaged across all plant species. Bars with asterisks indicate significant difference ($P < 0.05$, Dunnett one side test) with non-inoculated control (Nil) plants. $n = 15$ for each DSE isolate except in BG17 ($n = 13$) and PJ2 ($n = 14$).

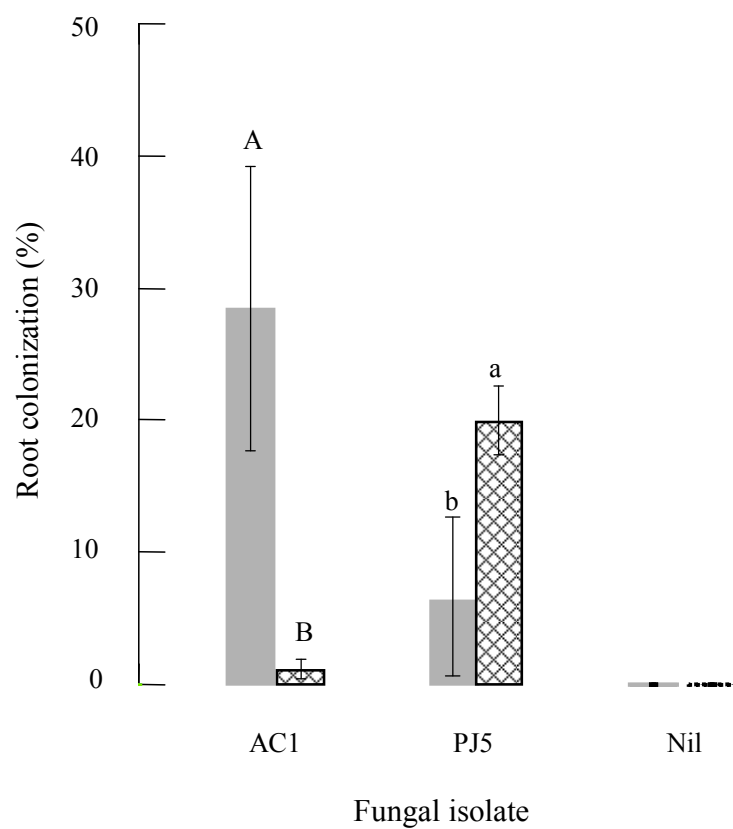
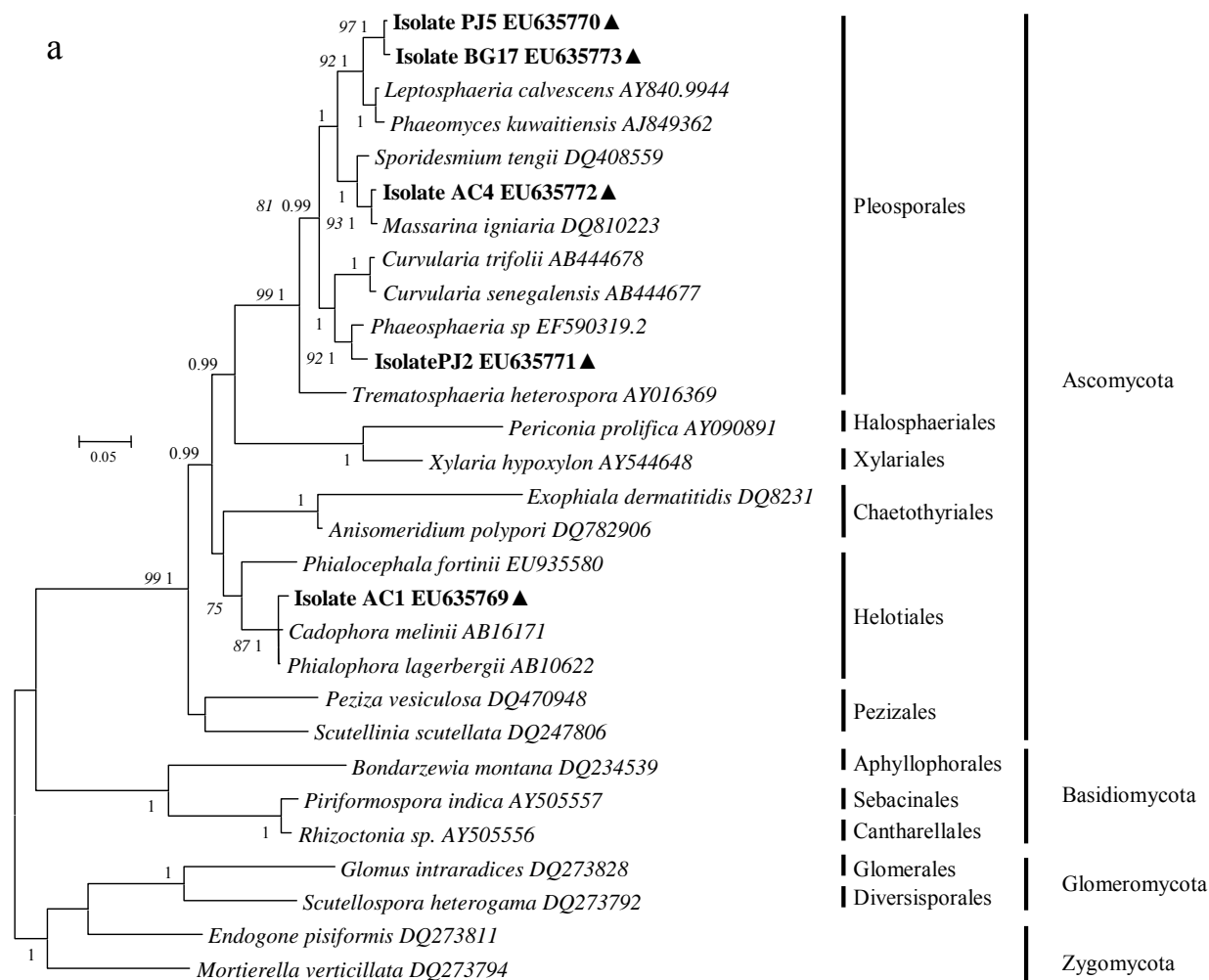


Fig. 3.3 Extent of root colonization by two isolates of dark septate endophytes in *A. cristatum* and *P. juncea*. Bars are least square means of three replicates with one standard error. Within each isolate, means with different letters are significantly different (contrast analysis, $P < 0.05$). Grey bars = *A. cristatum*, cross stacked bars = *P. juncea* ($n = 3$).



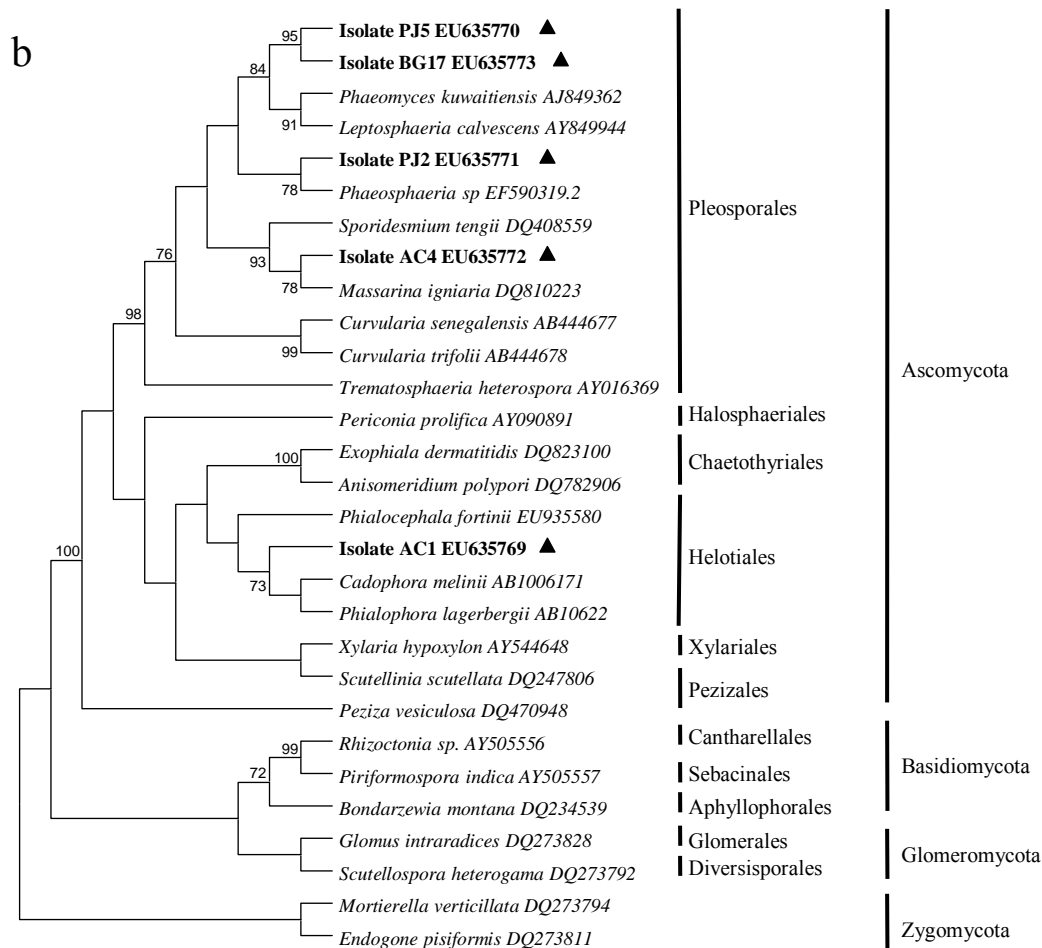


Fig. 3.4 Approximate identification of DSE isolates by phylogenetic analyses. The names and Genbank codes of the five isolates under study are in bold and followed by a solid triangle (▲). In italics are the codes and names of rDNA sequences of fungi downloaded from Genbank and referenced as root symbionts (Marquez et al., 2007; Summerbell, 2005) or included in the most complete fungal phylogeny published (James et al., 2006). *Mortierella verticillata* and *Endogone pisiformis* are used as an out group. **a**) Bayesian and Neighbor-Joining analysis. Italicized numbers in the branches are bootstrap values from 1000 iterations. Non-italicized numbers are posterior probabilities from MrBayes **b**) Maximum parsimony analysis tree. Numbers in the branches are bootstrap values from 500 iterations. Branch values higher than 0.95 in Bayesian analysis or 70 in bootstrap analysis are statistically significant (Hillis and Bull, 1993; Larget and Simon, 1999). Non significant values were omitted.

maintains effective photosynthesis when plants close their stomata for long periods of time to avoid water loss in hot and dry conditions (Hopkins and Hüner, 2003).

The DSE here, might have been a C drain providing little benefit to the already drought adapted *B. gracillis*. In contrast, the C investment into DSE symbioses by drought susceptible *A. cristatum* and *P. juncea* resulted in improved growth, apparently through plant cellular protection. Improved performance of symbiotic plants under drought was reported (Marquez et al., 2007; Rodriguez et al., 2008; Walker et al., 2003; Xu et al., 2008) and attributed at least partially to reduced oxidative cell damage, which commonly occurs in drought stressed plants (Rouhier and Jacquot, 2008). It was also found here that although these DSE colonize the roots of different plant species, they may show host preference and greater capacity to colonize the roots of the plant species from which they were isolated. It may be due perhaps to differences in root exudates composition (Biondini et al., 1988). As prior occupation of roots can deter another fungi to colonize (Alstrom, 2000), it is possible that the specificity between plants and DSE in the field may be greater than the observed here under controlled conditions.

Differential effect of DSE on grass growth or colonization in a plant community may influence its dynamics. For instance, isolate BG17, which reduced the growth of *B. gracillis*, was isolated from this grass species. In areas where BG17 exists, the fitness of *B. gracillis* would be reduced and that of co-occurring species benefited by the isolate, such as *A. cristatum* and *P. juncea* would be increased. Although it is also possible that DSE might improve the fitness of *B. gracillis* under more severe water shortage. *B. gracillis* with its C₄ metabolism might have experienced only mild drought stress under this experimental condition, as compared to the C₃ grasses included here.

Alternatively, in field grown *B. gracillis*, DSE might relieve the effect of growth limiting factors that were absent in this study. Disease resistance, winter hardiness, or production of grazer-detering molecules in plant tissues have been observed in presence of other grass endophytes that are sometimes parasitic (Faeth and Sullivan, 2003), but these effects were not tested in the current research.

Isolate PJ2 improved grass N nutrition ($P = 0.034$, Fig. 3.2), another effect that might be related to plant growth stimulation. Some DSE have improved plant growth and N nutrition by giving plant access to pools of organic N (Usuki and Narisawa, 2007; Zijlstra et al., 2005). However, there was no isotopic evidence supporting this hypothesis, and the reason for better N nutrition in PJ2 inoculated plants remains obscure.

The results of this research suggest that plant growth promotion and root colonization by DSE may be more specific than previously thought. Plant growth response was generally positive in the water stressed cool season grasses *A. cristatum* and *P. juncea*, and negative in *B. gracillis*. Intensity of root colonization by two DSE isolates was higher in the roots of the grass species from which they were isolated. It appears that grass symbiosis with DSE are highly specific, and may regulate the growth and perhaps the distribution of the grass species included in this research, mainly through drought stress reduction in *A. cristatum* and *P. juncea* and parasitism in *B. gracillis*. This hypothesis remains to be tested.

4 DEPTH DIFFERENTIAL MORPHOLOGY AND GENETIC DIVERSITY OF MYCORRHIZAL FUNGI IN PRAIRIE GRASSES

4.1 Abstract

Little is known on the genetic diversity of arbuscular mycorrhizal (AM) fungal communities in natural ecosystems and on the factors controlling this diversity, despite the role of AM in the productivity and stability of plant communities. I tested the impact of soil depth on root AM diversity, in 4 yr old pure stands of crested wheatgrass (*Agropyron cristatum*), switchgrass (*Panicum virgatum*), green needlegrass (*Nassella viridula*) and western wheatgrass (*Pascopyrum smithii*), growing in southwest Saskatchewan. Soil depth strongly influenced AM composition in all plant stands. A fine mycelium typical of AMF and a coarse and septate mycelium were observed in the roots. Shallow (3- to 15-cm depth) roots showed higher colonization than deeper roots and the coarse mycelium dominated root occupation at deeper depths (15- to 30 and 30- to 45-cm). Six phylotypes of the genus *Glomus* composed the AM community. Three were general colonizers while others were detected only in some grass species. AM phylotype diversity and richness was highest in shallow roots, except in *N. viridula* which hosted a larger number of phylotypes in deeper (30- to 45-cm) roots. AM phylotype composition always shifted with change in soil depth, and in roots of *P. virgatum* the rDNA sequences of one phylotype were segregated according to soil depth. It is concluded that grasses differ in their symbiotic strategy and that the environmental conditions encountered as roots grow in different soil horizons can be a mechanism contributing to the selection of AM communities and populations. Consequently, AM symbioses may be specialized to multiple niches across the soil profile.

4.2 Introduction

The symbiosis with AMF is ubiquitous in terrestrial ecosystems and usually provides plants with nutritional benefits and community stability. The diversity of AMF has been linked to plant

productivity and ecosystem stability (van der Heijden et al., 1988). Despite the cosmopolitan distribution of some species (Sanders, 2004) and the fact that any AMF can form symbioses with any host plant species, the specialization of AM taxa in specific soil conditions (Appoloni et al., 2008; Oehl et al., 2005) and plant hosts (Gollotte et al., 2004) was proposed. Such specialization in AMF should influence the structure of plant communities in different soil environments.

Due to the biotrophic nature of AMF, most knowledge on the biodiversity of these plant symbionts was gained through morphological observation of spores and roots extracted from the top 10 or 15 cm soil layer. This approach provided valuable knowledge on the ecology of AMF in soil plant ecosystems, but it has limitations. It can overlook AMF with sparse or periodical spore production (Rosendahl, 2008) or species (Oehl et al., 2005) adapted to deeper soil layers.

The diversity of AMF communities that might develop in different plant species or soil depths is important since differences in symbiotic functioning may exist within isolates of the same species (Munkvold et al., 2004) or between different genera or species of AMF (Dodd et al., 2000; Maherali and Klironomos, 2007). AMF communities at different soil depths might also respond differently to environmental conditions (Rillig and Field, 2003). However, little is known about the diversity of AM forms and functions in the Canadian prairies and on the influence of soil depth on AMF genetic diversity in plant roots. Understanding the spatial dimensions of AMF biodiversity can provide clues about the symbiotic strategies of prairie grasses, and the factors shaping the biodiversity of AMF communities and populations.

Environmental heterogeneity is an important condition to maintain AMF diversity (Koch et al., 2006). Sources of root environment heterogeneity in the Canadian prairies are common to other temperate grassland ecosystems, and include changes in plant phenology and succession of active plant species as the growing season progresses, as well as changes in soil the environment

as roots grow deeper and cross different soil horizons. Contrasting environments exist within the same plant rooting zone. Since arbuscular mycorrhizal fungi can be influenced by their plant (Rillig and Field, 2003) and soil environments (Dodd et al., 2000), soil depth may be a driver of AMF diversity.

The AMF are composed of coenocytic hyphae in which dissimilar nuclei are distributed (Hijri and Sanders, 2005). Understanding the genetic diversity in these haploid organisms for which no sexual stage is known has been challenging. Here, morphological, molecular and phylogenetic approaches were combined to describe the morphology of hyphae and genetic diversity of AMF in roots of prairie grasses at different soil depths. Specifically, I tested that the genetic diversity of AMF communities and extent of root colonization differs in four common grassland species, and that these symbiotic attributes vary with soil depth.

4.3 Materials and Methods

The colonization and community composition of mycorrhizal fungi at different soil depths was described in pure stands of switchgrass (*Panicum virgatum* L.), crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), green needlegrass (*Nassella viridula* Trin.) and western wheatgrass (*Pascopyrum smithii* (Rydb.) A. Löve). The long term (8 years) experimental plots were located at the Semiarid Prairie Agricultural Research Centre (latitude 50° 18' N; longitude 107° 41' W), near Swift Current, Saskatchewan, Canada.

The region receives an average of 361.4 mm of precipitation per year and has a yearly mean temperature of 3.6°C (54 yr average). About 50% of annual precipitation occurs between 1 May and 30 September and about 30% falls as snow in winter. Mean air temperature difference between July and January is about 33°C. The experimental plots were set in 2001 on a slight sloping (<3%) Orthic Brown Chernozem (Ayres et al., 1985), with pH of 6.51 and 6.59 (water saturated soil paste) and a silty loam and loamy texture in the A and B horizons respectively. The

experimental site was cropped with barley for four years before the establishment of the experiment in 2001, which was arranged in a complete randomized block design with four replicates. Each plot was 6 by 4 m.

4.3.1 Root sampling and processing

One or two soil cores 5-cm wide and 45-cm long were extracted three times between October of 2005 and August of 2006 from each of four replicated plots. To avoid sample contamination with surface transported materials, the first three centimeter layer of soil and any external root were removed before cutting cores in segments containing the top 3- to 15, 15- to 30 and 30- to 45-cm depth soil layers. The roots were extracted by hand, pooled into the same plot and depth and gently washed over a 100 mesh sieve under running tap water. Then roots wrapped in a nylon mesh and attached to a styrofoam floater were placed during 5 min in an ultrasonic cleaner (FS 30, Fisher scientific, Pittsburgh), dried with sterile towel paper and finally cut into 1 cm pieces with a sterile scalpel. The root samples were cleaned within 48 hours after harvest, and stored at -12°C until analysis.

4.3.2 Root colonization by AMF

The percentage of mycorrhizal colonization in October of 2005 was obtained from 48 root samples (four plant stands \times four replicates \times three depths [3- to 15, 15- to 30 and 30- to 45-cm depth]), by previous clearing in 10% KOH and further staining in 5% Schaeffer black ink in vinegar (Vierheilig et al., 1998). Colonization assessment was made at 400X magnification under a compound microscope using the line intercept method (Giovanetti and Mosse, 1980). Two types of mycelia were scored: a) a fine mycelium of about 3 μm in diameter usually associated with arbuscules and vesicles, and b) a coarse, sparsely septate, coil forming mycelium of about 8 μm in diameter.

4.3.3 Community composition of AMF at two soil depths

The community composition of AMF in each plant stand was obtained from 32 composite samples (4 plant stands x 2 depths x 4 replicates per depth) obtained by pooling roots sampled in May and August of 2006, and further cloning and sequencing of the 5' end of the long subunit of ribosomal genes (LSU rDNA).

Fifty mg of roots from each composite sample were freeze dried and milled with a tungsten bead in a micro centrifuge tube by vigorously shaking for three min in a paint shaker. The DNA was extracted using the DNAeasy plant mini kit (Qiagen Inc.), according to the manufacturer's instructions, and stored at -20°C until use.

Amplification of DNA followed a nested approach. A first PCR reaction with primers LR1 [5'GCATATCAATAAGCGGAGGA3'] (Van Tuinen et al., 1998) and FLR2 [5'GTCGTTTAAAGCCATTACGTC3'] (Trouvelot et al., 1999) amplified the 5' end of the LSU rDNA of general fungi. Ten µL of PCR cocktail contained 5 µL of AmpliTaq Gold PCR master mix (Applied Biosystems), 3.8 µL of ultra pure water, 0.1 µL of a 50 µM solution of each primer and 1 µL of template DNA. The amplification included one step at 95°C for 10 min, followed by 35 cycles of 1 min at 93°C, 1 min at 60°C, 1 min at 72°C and a final step of 10 min at 72°C. The second PCR reaction with primers LR1 and FLR4 [5' TACGTCAACATCCTTAACGAA 3'] (Gollotte et al., 2004), amplified the 5' end of the LSU rDNA of AMF. Amplification conditions were as described above, except that 1 µL of a 1/500 dilution of PCR product from the first reaction was used as a DNA template. PCR products were analyzed by agarose gel electrophoresis for the presence of an 800 bp band of DNA. After the second PCR cycle, one composite sample of PCR products was made from the four replicates of each depth/plant species, and 1.2 µg of this DNA was cloned using a Topo TA cloning kit for sequencing (Invitrogen Corp) resulting in one clone library for each depth in a given plant

species. This pooling procedure does not reduce the detection of AMF (Renker et al., 2006), but reduced to $\frac{1}{4}$ the number of cloning reactions. Positive transformants containing a fragment of DNA of about 800 bp were screened by PCR with primers LR1-FLR2, and 345 plasmids were sequenced with primers T3-T7 at the Plant Biotechnology Institute in Saskatoon (National Research Council, Saskatoon, SK, Canada). The rDNA sequences presented in this research were deposited in Genbank under codes EU379972 to EU380188.

4.3.4 Alignment and check for chimera rDNA sequences

The sequences were aligned in Clustal W with gap opening and extension penalties of 6 and 3 respectively. Chimera sequences formed during the PCR amplification (Wang and Wang, 1997) were removed by re-submission of the alignment to the software Bellerophon (Huber et al., 2004), until no chimera sequences were detected.

4.3.5 Overall structure, phylogenetic classification and distribution of AMF phylotypes across plant stands and depths

The overall structure of the AMF community was obtained from the computation of similarity values between all non-chimera rDNA sequences, and further visualization by multidimensional scaling (MDS). The procedure followed the computation of a matrix containing square pair wise differences [D]. Then a proximities or similarity matrix [S] to use in the MDS analysis was obtained by subtracting [D] from its highest value of dissimilarity (dmax) according to: $[S] = d_{\max} - [D]$ (Legendre and Legendre, 1998). [D] and [S] were computed in the software Arlequin 3.11 (Excoffier et al., 2005) and MS Excel® respectively. The MDS configuration was obtained from a Guttman loss function in the software Systat 12.

Approximate phylogenetic identification of AMF was obtained by Bayesian, distance and parsimony based analysis. Bayesian and Neighbor-Joining analysis used sample sequences from each cluster obtained by MDS along with 32 sequences representing all families of known

Glomeromycota, and two sequences of the genus *Mortierella* included as an out group, and aligned as described above. The consensus phylogenetic tree was obtained from two independent runs of MrBayes 3.11 (Huelsenbeck and Ronquist, 2001) using a GTR + G model, 1,000,000 generations, 35% burnin time and tree sampling each 500 generations. Additional confidence was obtained from bootstrap analysis of a Neighbor-Joining tree built with JC genetic distances and gamma rates of nucleotide substitution in the software MEGA4 (Tamura et al., 2007). Models of nucleotide substitution for both trees were selected using the software PhyML (Guindon and Gascuel, 2003). In parsimony analysis, a consensus from the most parsimonious trees was calculated using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 519 positions in the final dataset, out of which 224 were parsimony informative. Parsimony analysis used only part of the sequences used for Bayesian and Neighbor joining analysis, due to computational limitations of this method (Tamura et al., 2007).

Support for the branches is presented as posterior probabilities in MrBayes or bootstrap values from 1000 and 500 iterations in Neighbor-Joining and Maximum Parsimony analysis. Values lower than 0.95 using Bayesian posterior probabilities (Larget and Simon, 1999) or 70% in bootstrap analysis (Hillis and Bull, 1993) are considered non-significant. Since no formal definition of species exists for phylogenetically identified AMF individuals based on LSU rDNA analysis, AMF phlotypes are presented here as aligned sequences sharing more than 81.6% similarity estimated from pair wise nucleotide differences, which clustered together in significantly supported clades or within known species of AMF in the phylogenetic analysis. Further detailed distribution of AMF species was obtained by partitioning the matrix of DNA

sequence similarity used above for MDS analysis, according to plant stands and root sampling depths, and drawing new MDS plots for each grass stand.

4.3.6 Rarefaction and diversity index estimates

Species accumulation curves and Shannon diversity indices were computed to know if the number of clones sequenced adequately represented the richness of AMF species, and to compare the relative diversity of AMF species at each depth. Standard deviation values in the phylotype accumulation curves or Shannon diversity indices were estimated from 1000 resamplings without or with replacement respectively, using the software Estimates (Colwell, 1997).

4.3.7 Assessment of treatment effects

Values of root colonization by the fine mycelia were transformed before analysis to meet the assumption of normal distribution. Two way repeated measures ANOVA was used to estimate the effect of sampling depth, plant species or their interaction on values of root colonization by each type of mycelia. A significant effect of soil depth on root colonization was followed by comparisons with Bonferroni tests. Statistical analyses were computed in the software SAS.

Differences in composition of AMF communities between depths in each plant stand were tested by a permutation approach. Briefly, matrices containing Jukes-Cantor genetic distances were calculated with rDNA sequences from each depth in the program DNAdist in Phylip, and loaded in the software J-LIBSHUFF (Singleton et al., 2001). A P value was obtained from 1000 permutations, Bonferroni corrected according to the number of comparisons, and used to assess significant differences between AMF communities at each depth in a given plant stand. This test is asymmetrical, therefore the AMF population at two soil depths A and B can be significantly different, whereas B versus A is not; this would indicate that the species composition of population B is likely to represent a subset of population A (Singleton et al., 2001).

4.4 Results

4.4.1 Root colonization by AMF

Root colonization by both types of mycelia was significantly higher ($P < 0.01$) in shallow than in deeper root samples, and decreased with soil depth. Although the coarse mycelia dominated root colonization in deeper soil layers (Fig. 4.1). The effect of plant species or their interaction with soil depth was not significant.

4.4.2 Community composition of AMF in different grass stands and root sampling depths

The distribution of AMF phylotypes across plant stands and soil depths was obtained from 217 sequences after removing 128 suspicious chimera sequences. The MDS analysis grouped the sequences in six clusters (Fig. 4.2a), which were well supported as six AMF phylotypes of the genus *Glomus* (Fig. 4.2b). These AMF phylotypes are referred later as *Glomus intraradices*, *Glomus clarum* or Glo3 to Glo6.

The overall community was dominated by phylotypes Glo5 and *G. intraradices*, which accounted for 59% and 22% of the rDNA sequences obtained, respectively. Phylotypes *Glomus clarum*, Glo3, Glo4 and Glo6 were less frequent and accounted for the remaining 19% of the sequences obtained (Fig. 4.3).

Each plant species hosted a distinct community of AMF, despite the presence of some generalist phylotypes. In all plant species, the AMF communities varied with depth ($P < 0.025$) and the occurrence of some AMF phylotypes at a given depth depended on the plant species analyzed. For example, Glo5 or *G. intraradices* were common colonizers of all plant species (Fig. 4.3), but Glo5 was undetected in the shallow roots of *N. viridula* and *G. intraradices*, was undetected in the deep roots of *P. virgatum* (Fig. 4.4). Glo4 occurred only in shallow roots of *P. virgatum* and deep roots of *N. viridula* (Fig. 4.4).

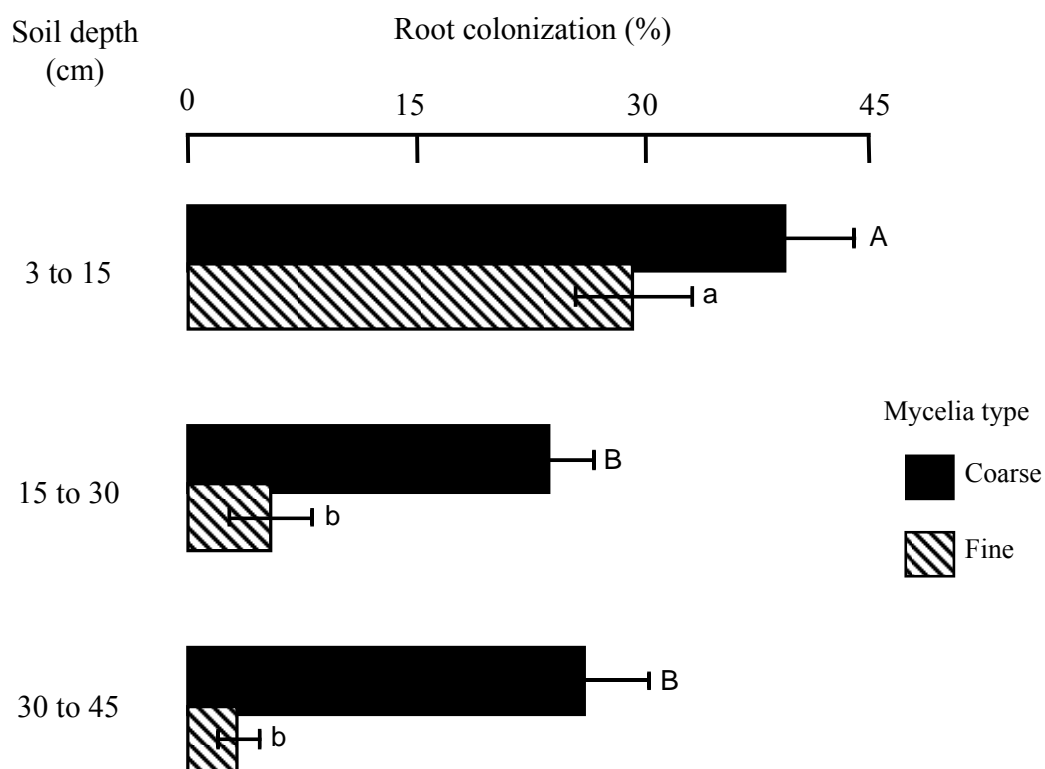
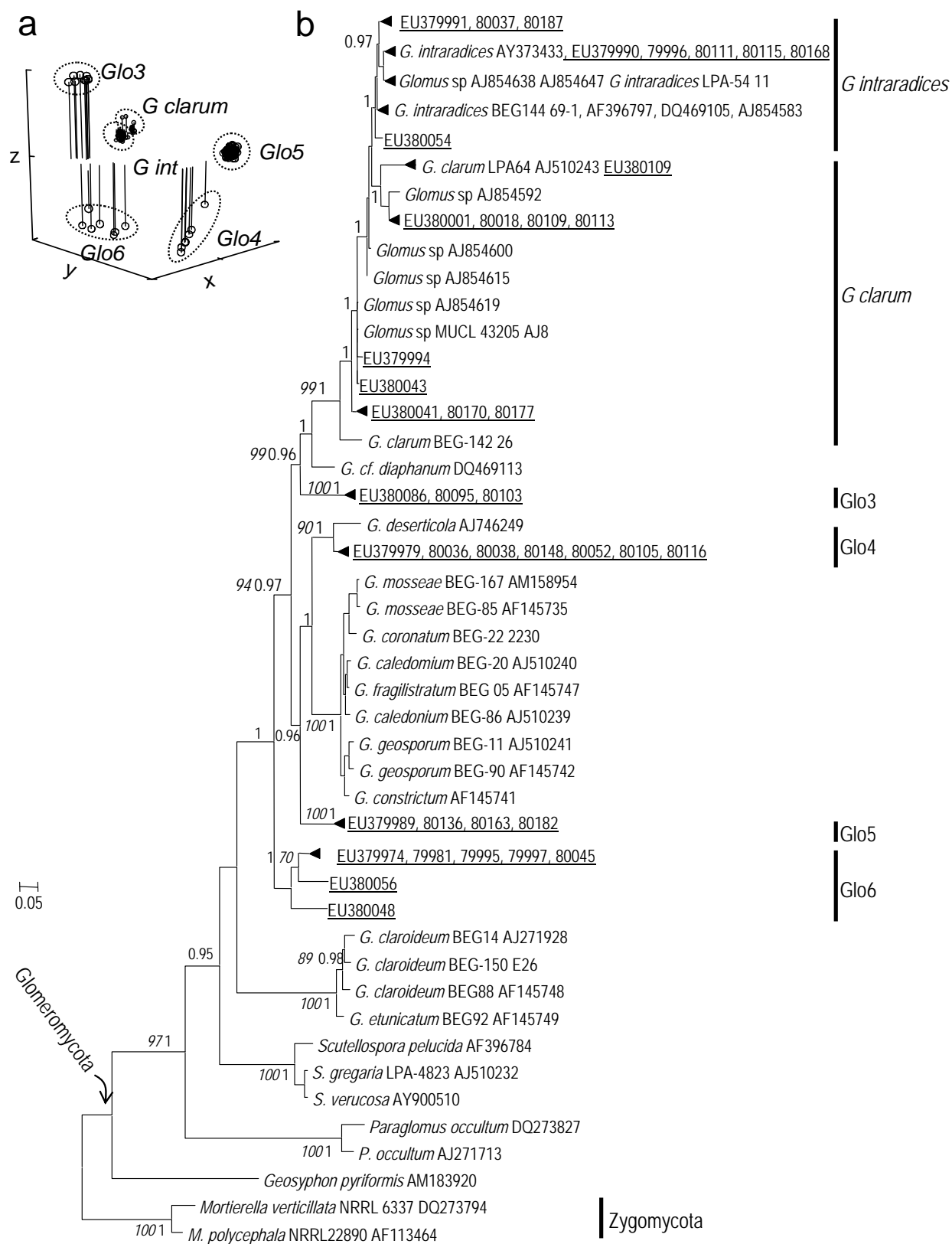


Fig. 4.1 Colonization by two types of AMF mycelia in roots of prairie grasses sampled from three soil depths. Bars are least square means of values of root colonization averaged across all plant species, with their standard error. At each soil depth, capitalized letters are used to compare root colonization by the coarse mycelia; non capitalized letters compare root colonization by the fine mycelia (Boferroni test, $P < 0.05$, $n = 16$).



C

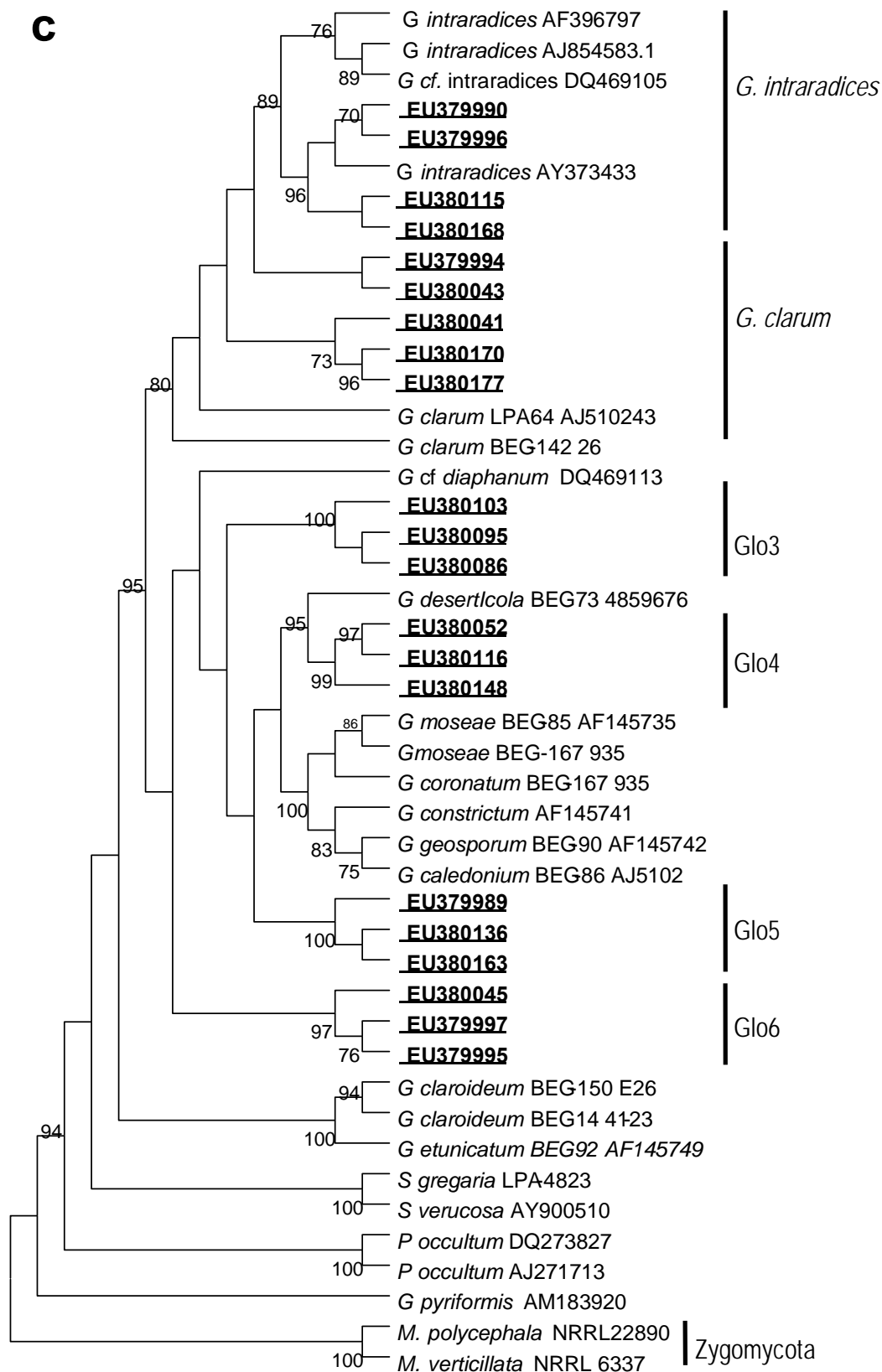


Fig. 4.2 Structure and phylogenetic classification of the AMF community **a)** Overall structure of the AMF community based on similarity values between all LSU rDNA sequences, as revealed by multidimensional scaling analysis. Each circle in the scatter plot represents a sequence of rDNA and circles closer to each other represent more similar rDNA sequences. Vertical sticks to zero in axis z are added to aid in visualizing clusters, and solid circles appear by overlapping of highly similar sequences (Stress = 0.041). **b)** Phylogenetic analysis of AMF rDNA sequences obtained from this research (underlined) or downloaded from Genbank. All sequences from Glo4 and Glo6; or sample sequences from Glo1, Glo2, Glo3 and Glo5 in the MDS plot were used in the tree. Non-italicized numbers in the branches are posterior probabilities from the Bayesian tree. Numbers in italics are bootstrap values from 1000 iterations in a Neighbor Joining analysis. **c)** Maximum Parsimony analysis. In bold and underlined are Genbank codes to identify rDNA sequences from this research. Numbers in the braches are bootstrap values from 500 iterations. Values higher than 0.95 or 70 for Bayesian or bootstrap analysis are significant. Only significant values are shown.

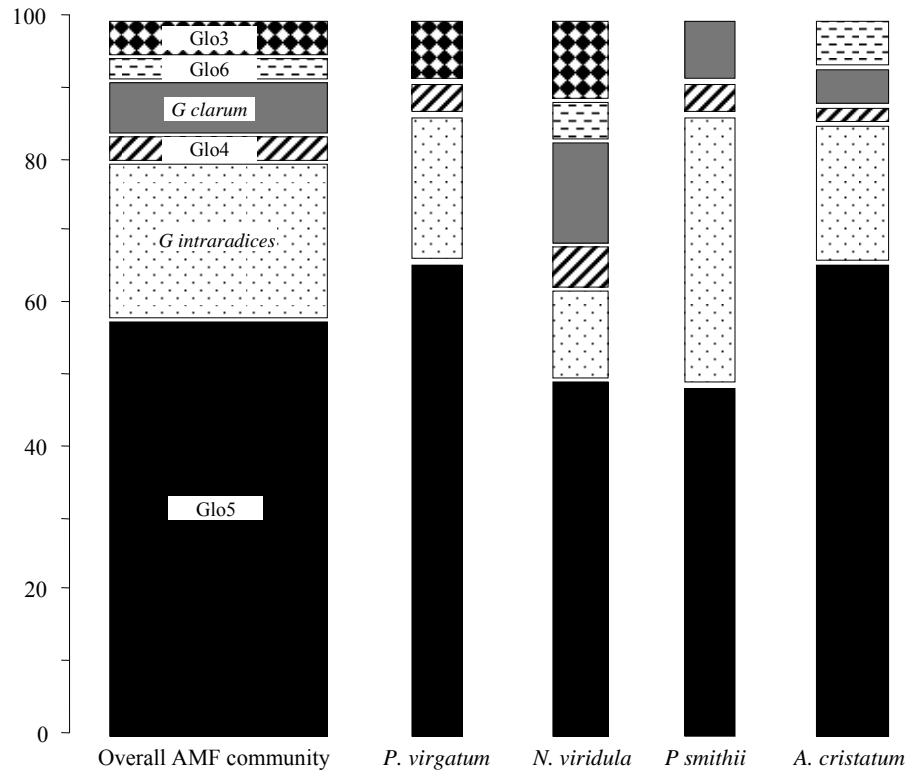


Fig. 4.3 Overall compositions of the AMF community or communities detected in each grass stand. Labels for compartments in each plant species are as in the widest bar. The width of a bar is proportional to the size of the clone library constructed ($N = 217$ for the overall AMF community).

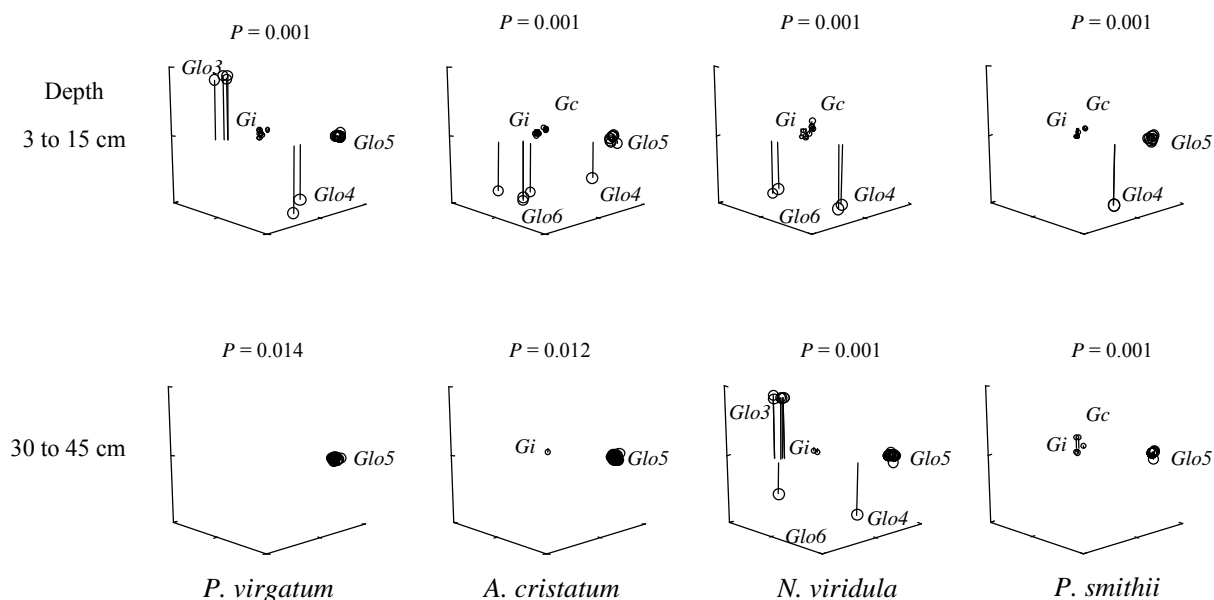


Fig. 4.4 Composition of the AMF community in roots of different prairie grass species sampled at two soil depths. Each circle in a scatter plot represent a sequence of AMF rDNA, and the distance between circles is proportional to the degree of similarity between the sequences that they represent. Labels indicate AMF phylotypes (Gi= *G. intraradices*, Gc= *G. clarum*). Values of P above a scatter plot compare one AMF community to its counterpart in the other depth and they are significant if $P < 0.025$ (Bonferroni correction). From left to right, $n = 28, 30, 17$ and 29 (3- to 15-cm depth); $22, 32, 38$ and 21 (30- to 45-cm).

In *P. virgatum*, differences in AM composition at different depths were induced by apparent genetic variation within isolates of a same AMF phylotype. rDNA sequences of Glo5, the only phylotype detected in deeper root samples, were not a subset of those in shallow root samples ($P = 0.012$, Fig. 4.4), which also included Glo5. However, in *N. viridula* and *P. smithii*, differences between shallow and deeper AMF communities were induced by a shift in AM phylotypes distribution (Fig. 4.4).

The diversity and richness of AMF species in roots was generally higher at shallow depth, except in *N. viridula*, which hosted higher richness of AMF at deeper depth (Figs. 4.4 and 4.5). The species accumulation curve in deep roots of *N. viridula* did not reach a plateau (Fig. 4.5), suggesting that the number of clones sequenced did not adequately represent the diversity of its AMF community, although the sequencing effort was adequate in other plant species

4.5 Discussion

This research found a large influence of soil depth on fungal occupation of roots in all grass species examined and confirmed earlier reports of declining hyphal density with increasing soil depth (Abbott and Robson, 1991; Jakobsen and Nielsen, 1983). It also detected a large influence of soil depth on the genetic diversity of AMF in grass roots, which was modified by the host plant species. The distribution of AM phylotypes revealed the existence of plant and depth specific AMF communities. rDNA sequences of phylotype Glo5 were also segregated according to soil depth in roots of *P. virgatum*. These results reveal a selective effect of soil depth on the genetic make-up of AMF communities and populations in the roots of prairie grasses, and shed new light on the process of natural selection in these multigenomic fungi. They also suggest the existence of different AM symbiotic strategies, which may be involved in plant community dynamics in grasslands.

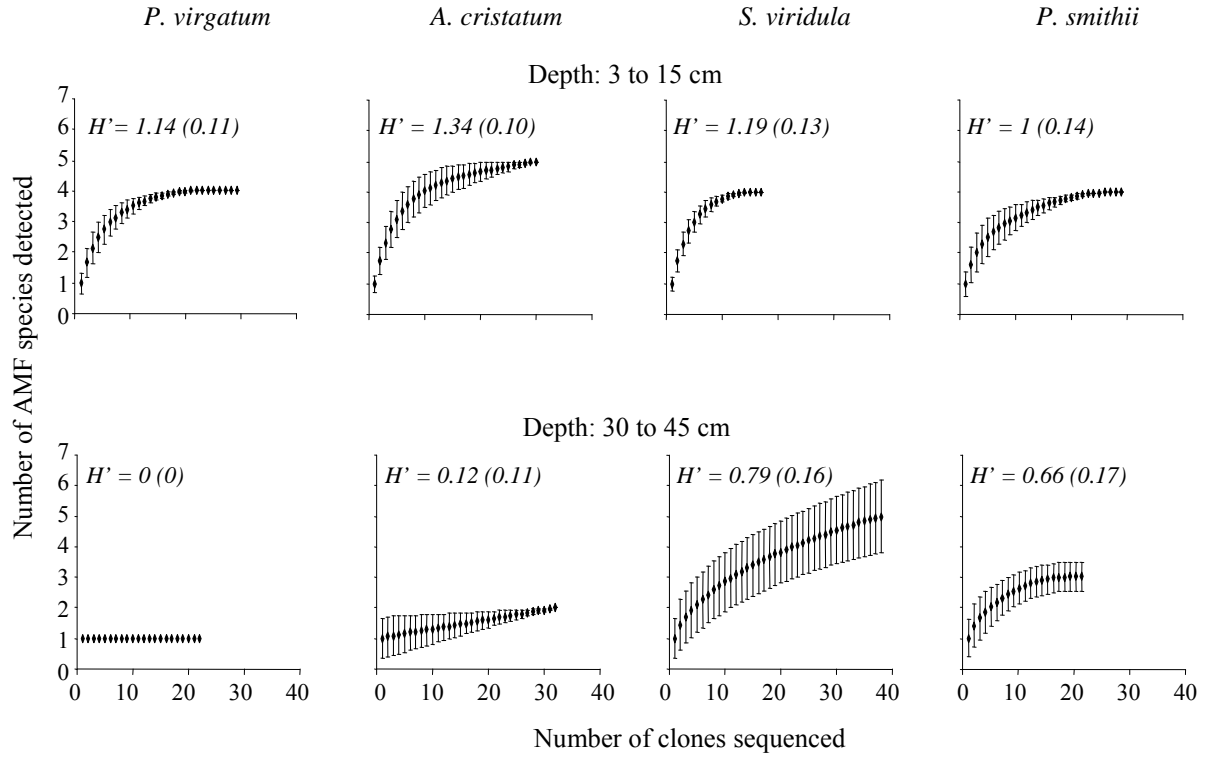


Fig. 4.5 Estimates of richness and diversity of the AMF community in roots of prairie grasses sampled from two depths, as a function of the number of clones sequenced. Each point in the curves represents the mean number of expected phylotypes ± 1 standard deviation. H' is the Shannon index of diversity with the standard deviation given in parenthesis. All parameters were estimated from 1000 resamplings.

The AMF genetic differentiation in response to soil depth presented here may be explained by formerly proposed models of heterogeneous genetic organization in AM mycelia. It is known that a single spore of AMF contain genetically distinct nuclei (Hijri and Sanders, 2005) and it is probable that the functionality of the multigenomic hyphae they produce is not spatially fixed (Sanders 2002). Different AMF hyphae can anastomose (Giovannetti et al. 2004; Hamel, 2007; Sanders, 2002), and recent findings indicate that these anastomoses allow different nuclei to mix and generate hyphae containing nuclei with a new set of genetic information (Croll et al., 2009). It may be possible that certain nuclei or combinations of nuclei in an AM mycelial network are better adapted to some soil environmental conditions, resulting in the selection of nuclei in different parts of this network, and perhaps in the creation of AMF networks with spatially variable functionality (Hamel, 2007). Anastomosis appears as a mechanism allowing parts of an AM network to acquire genetic information from a pool that can increase its fitness as it grows in a new soil environment.

The present study provides field evidence supporting the concept of AM network heterogeneity, although it also brings up a question previously raised (Rosendahl, 2008): if different nuclei (and ribosomes) segregate in different points of the same mycelial network, how can an AMF synchronize the synthesis of its proteins? Furthermore, do AM mycelia parse into different isolates after hyphal anastomoses? If so, under which soil conditions? These are fundamental, but unanswered questions about the biology of this important group of fungi, which require further scrutiny. Population genetics tools applied to field studies and examination of hyphal network continuity in field samples should provide more insight on the evolutive forces shaping this diversification and functioning of AMF in the field.

The specific AMF community assemblage found in each grass species and at each depth suggests that different grasses might have different symbiotic access to resources along the soil profile. This is possible since different AMF species or isolates within a same species may possess different symbiotic attributes, for example, different P adsorption, plant growth promotion or mycelial growth capacities (Koch et al., 2006; Maherali and Klironomos, 2007; Munkvold et al., 2004). It appears that *P. virgatum* exploits preferentially the top soil layer, whereas other grasses have better access to deeper soil resources, since they are associated with a more diverse community of AMF at deeper depth. This would explain relative fitness differences among the grasses studied under this particular environment.

The experimental approach employed here captured most of the AMF diversity present in the grasses studied, but important questions regarding the identity of the coarse mycelia also remain. The morphology of these coarse and septate mycelia, which dominated root colonization in the grasses, does not correspond to that of AMF mycelia. Fine and coarse mycelia present in roots have often been classified as Arum and Paris types of AMF mycelia, after a description presented more than 100 years ago (Dickson et al., 2007). But in these cases the mycelia were associated with typical AMF structures such as arbuscules or coils, which were not observed here. It is important to confirm the identity of these different mycelia which were differentially distributed in depth, and to know if different mycelia, which can be generated by the same AMF species (Barrett, 1958) possess the same genetic material. The mycelium is the functional unit in AMF symbiosis, and mycelia morphological differences are associated with differences in symbiotic functionality (Dodd et al., 2000). Different types of AMF mycelia have also triggered different plant defense responses during symbiotic interactions (Gao et al., 2004), and expressed different phosphorus-to-carbon exchange ratios (Pearson and Jakobsen, 1993). In addition, since

fungus spores represent a resting stage, it is possible that mycelia in soil and roots provide the phenotypic expressions in which natural selection can act to generate new AMF isolates and species. The use of molecular tools directly on mycelial networks should provide more light on these aspects of AMF ecology and evolution.

In the grasses studied, soil depth was a strong driver of root colonization and composition of AMF communities and populations. The diversity and richness of AMF phylotypes as well as root colonization were generally higher at shallow soil depth. There were depth specific assemblages of AMF communities, and community structure also depended on the associated host plant. While some AMF were clearly general colonizers, others showed preference for a depth or a host plant species. These results strongly suggest the existence of niche specialization in AMF along the soil profile, which is influenced by the host plant. The existence of specific plant and depth AM assemblages suggests the presence of differential symbiotic strategies in prairie grasses.

5 DYNAMICS OF ARBUSCULAR MYCORRHIZAL (AMF) AND DARK SEPTATE ENDOPHYTIC (DSE) FUNGAL COMMUNITIES COHABITING IN ROOTS OF PRAIRIE GRASSES AS REVEALED BY TRFLP ANALYSES

5.1 Abstract

Dark septate endophytic fungi (DSE) and arbuscular mycorrhizal fungi (AMF) co-occur in plant roots in many ecosystems. Despite their potential role in plant and ecosystem processes, little is known about the dynamics of these symbioses in relation to plant hosts or environmental conditions. In this research, changes in root DSE and AMF community composition in the A and B horizons of the soil were followed during one growing season in pure stands of switchgrass (*Panicum virgatum* L.), crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), green needlegrass (*Nassella viridula* Trin.) and western wheatgrass (*Pascopyrum smithii* (Rydb.) A. Löve), in southwest Saskatchewan. DSE and AMF community composition in grass roots changed during the growing season and the pattern of that change was different for DSE and AMF. Temporal changes in these communities were due to change in relative abundance of DSE and AMF phylotypes which proliferated at specific sampling times or soil depths. The DSE communities of roots are different at the beginning and end of the growing season, whereas AMF communities are not, suggesting a more dynamic behavior in DSE root colonization. DSE and AMF community structures were positively correlated, and independent of soil temperature, depth or grass species. Although DSE and AMF phylotypes generally appear to be mutually exclusive, two DSE phylotypes were associated with the AMF community. The results suggest the existence of niche separation in time and space in prairie grass roots, which could promote the development of transient and interacting symbioses between grass roots and DSE and AMF. It is proposed that these transient symbioses could be a mechanism which extends plant use of

nutrients and provide plant tolerance to stressful conditions appearing at different times during the growing season.

5.2 Introduction

Plant symbioses with dark septate endophytic (DSE) and arbuscular mycorrhizal fungi (AMF) are widespread in terrestrial ecosystems, and the role of these symbioses on plant adaptation to stressful conditions is being discovered. The importance of AMF symbioses had been mainly related to plant nutrition, particularly in nutrient poor environments. AMF extraradical hyphal networks reach soil nutrients unreachable by the root alone. Then hyphae absorbed nutrients are transferred to plants in intraradical symbiotic interfaces (Brundrett, 2004). DSE symbioses may also provide nutritional benefits to plants (Mullen and Schmidt, 1993), although the most striking role found among this taxonomically diverse, melanized fungi is to improve plant growth under extreme soil salinity (Waller et al., 2005) or high temperature (Marquez et al., 2007). Despite their co-occurrence in roots, each fungal group is usually studied separately.

In few studies in which DSE and AMF root colonization was studied together, microscopic analysis were used to track hyphal characteristics typical to each fungal group (Lingfei et al., 2005; Mandyam and Jumpponen, 2008; Medina-Roldan et al., 2008; Mullen and Schmidt, 1993; Muthukumar et al., 2006; Schmidt et al., 2008; Upson et al., 2008). These reports suggest that simultaneous colonization of plant roots by DSE and AMF is common throughout a range of environmental conditions, and that seasonal root colonization by DSE and AMF has different dynamics (Mandyam and Jumpponen, 2008). It is also apparent that in grassland ecosystems, colonization by DSE may be more abundant in roots than colonization by AMF (Medina-Roldan et al., 2008).

knowledge about the ecology of co-occurring DSE and AMF symbiosis was found from microscopic analysis of roots, but the resolution of this approach is limited. DSE (Sieber and

Grünig, 2005) and AMF (Sanders, 2004a) show remarkable genetic diversity that can not be revealed by morphological traits, and it is not possible to infer from microscopic observations if variations in abundance of DSE and AMF depends on specific individuals, or is the result of the activity of the entire communities. Differences in fungal root endophytes community composition are relevant since they can be related to differences in symbiotic functioning (Munkvold et al., 2004), adaptation to different hosts (Gollock et al., 2004) or overall environmental conditions.

The environmental limits for DSE and AMF functioning appear to be different. Several DSE appear to tolerate extreme soil temperatures (Kytöviita, 2005; Redman et al., 2002; Schmidt et al., 2008; Upson et al., 2008), salinity (Waller et al., 2005), and pH values (Postma et al., 2007) better than AMF. Each fungal group is usually studied independently. However, the wide co-occurrence of DSE and AMF symbioses in roots and the presumed access to a common pool of plant synthesized organic compounds suggest that co-occurring DSE and AMF symbiosis should be studied together. It is not known if DSE and AMF compete for plant supplied C, but competing interactions could lead to spatial and functional separation of fungal groups (Gadgil and Gadgil, 1971). Such functional separation appears to be common in rhizosphere associated fungal communities (Dickie et al., 2002; Lindahl et al., 2007). Although the causes of functional separation of microbial groups is not understood, it might be important for resource partitioning and stability of mixed plant communities through microbially mediated niche separation (Genney et al., 2000; Reynolds et al., 2003).

It is apparent that diverse and complex interactions between DSE and AMF may take place at a given point in time in the roots. Although DSE and AMF appear to be adapted to different environments (Kytöviita, 2005), members of both groups appear to be able to adapt to extreme

conditions. Therefore, these interactions between DSE and AMF should be more complex in seasonal and heterogeneous environments in which they co-occur. The prairie ecozone is characterized by a seasonal succession in vegetation and environmental conditions. Good water availability and a landscape dominated by cool season C_3 vegetation early in the season, are in contrast with the high temperature and prolonged drought periods during which C_4 and drought adapted plants may remain active during the late summer portion of the growing season. The change in climate and vegetation is accompanied by dramatic changes in root exudation within the same plant species (Henry et al., 2007) or in the quality of sugars generated by C_3 and C_4 photosynthesis (Henn and Chapela, 2000), which might in turn affect fungal community composition or metabolism. Environment variability also occurs with changes in soil depth. Prairie grasses commonly possess a deep root system that crosses soil layers differing in physico-chemical properties which exposes its endophytes to multiple environmental conditions. Under this succession of contrasting environments occurring in prairie grass roots, it is unlikely that the same root endophytes occur at all times during the growing season. Therefore, I tested the general hypothesis that fungal root endophyte composition changes during the growing season.

Several molecular methods had been used to study root endophytic communities. In particular terminal restriction fragment length polymorphism (TRFLP) had been used to study microbial communities and their variation across environmental gradients (Singh and Thomas, 2006). Compared to other community analysis methods, TRFLP has higher resolution and provides reproducible results (Singh and Thomas, 2006). The combination of TRFLP analysis and cloning of DNA makes it possible to track microbial species in natural environments (Campbell et al., 2004). In that approach, reference terminal restriction fragments (TRFs) are generated either from computer simulated restriction digestion of DNA sequences, or from the digestion of

cloned DNA. Samples of interest are analyzed by comparing the length of TRFs in digested DNA, to the length of these reference TRFs (Campbell et al., 2004).

Pure cultures of various DSE and cloned AMF rDNA were obtained from roots of several grass species common to the Canadian prairie ecozone, as reported in Chapters 3 and 4 of this thesis. Primer sets which simultaneously amplify the rDNA of these DSE and AMF were also identified (Appendix). These fungal cultures, cloned AMF rDNA and primer sets were used to develop reference TRFs specific to these DSE and AMF present in grass roots, in order to track by terminal restriction fragment length polymorphism (TRFLP) analysis, temporal and spatial changes in DSE and AMF community composition, and to explore any possible relationship between DSE and AMF community structure. Here, results from TRFLP analysis of grass roots from the A and B horizons of the soil at three sampling times during the growing season, show spatial and temporal changes in DSE and AMF community composition during the growing season and the relatedness of DSE and AMF community structure

5.3 Materials and Methods

5.3.1 Experimental site and plant stands

The dynamics of DSE and AMF communities at two soil depths was studied in the roots of switchgrass (*Panicum virgatum* L.), crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), green needlegrass (*Nassella viridula* Trin.) and western wheatgrass (*Pascopyrum smithii* (Rydb.) A. Löve), growing in southwest Saskatchewan. The long term (8 years) experimental plots sampled for this study were located at the South farm of the Semiarid Prairie Agricultural Research Centre (latitude 50° 18' N; longitude 107° 41' W), near Swift Current, Saskatchewan, Canada.

The region receives an average of 361.4 mm of precipitation per year and has a yearly mean temperature of 3.6°C (54 years average). About 50% of the annual precipitation occurs between

1 May and 30 September and about 30% falls as snow in winter. Mean air temperature difference between July and January is about 33°C. Mean soil temperature averaged across the A and B horizons during the sampling period of this study was 12, 24 and 11°C on May 22, August 7 and October 15 of 2007. The experiment was set in 2001 on a slight sloping (<3%) Orthic Brown Chernozem (Ayres et al., 1985), with pH of 6.51 and 6.59 (saturated soil paste) and a silty loam and loamy texture in its A and B horizons respectively. The experimental site was cropped with barley for four years prior to the establishment of the experiment, which was planted with the experimental grasses in 2001 in a complete randomized block design with four replicates. Each plot was 6 by 4 m in area.

5.3.2 Root sampling and processing

Two soil cores, 5 cm in diameter and 45 cm long were extracted from each plot on May 22, August 7 and October 15 of 2007 using a motorized coring device. The top three cm layer of the soil and any root external to the core were removed before separating the A and B horizon portions of the cores. Inner roots were removed by hand and pooled to create one root sample for each horizon of each plot. The samples were thoroughly washed over a 100 mesh sieve under running tap water, wrapped in a nylon mesh and attached to a piece of styrofoam floater, which supported the samples during two five minute periods in a sonication cleaner (FS 30, Fisher scientific, Pittsburgh) filled with reverse osmosis filtered, autoclaved water (130°C, 30 min, 0.138 MPa). Fresh sterile water was used for each sonication period. Root samples were dried in sterile paper towels, cut into 0.5 cm long pieces with alcohol swiped scissors, and freeze dried during 48 hours. All samples were processed within 24 hours after harvest and stored at -12 °C until analysis.

5.3.3 Design of reference terminal restriction fragments (TRFs) for analysis of DSE and AMF

The DNA of DSE and AMF present in the roots of the experimental grasses was obtained and sequenced as described in Chapters 3 and 4 of this thesis. Detailed information about these DNA materials is presented in Table 5.1.

Computer simulated digestion of AMF and DSE rDNA sequences was used to select candidate restriction enzymes, with basis on the generation of TRFs in a range of 30 to 400 bases long, and enough length variability to discriminate most DSE and AMF phylotypes. The software `t_DistinctiEnz` (http://www.bioinformatics.org/~docreza/cgi-bin/restriction/t_DistinctiEnz.pl) was used to generate theoretical TRFs at the 5' and 3' strand ends of three DSE and 34 AMF partial LSU rDNA sequences (Table 5.1). Enzymes Taq I and Sau 96I at 5' ends were selected among 231 restriction enzymes available in `t_DistinctiEnz`, as the most suitable to discriminate the DSE isolates or AMF phylotypes of interest, and were tested *in vitro*.

In vitro tests with TaqI and Sau96I included eight samples of AMF rDNA previously cloned in bacterial cells that were randomly selected from a 217 clone library, along with 15 samples of genomic DNA obtained from DSE fungal cultures (Table 5.1). This DNA samples were PCR amplified using the AMF specific primer pair 5'FAM LR1-FLR4, or the general fungal primers 5'FAM LR1-FLR2. Routines for DSE isolation, DNA extraction, PCR amplification and DNA cloning are presented in Chapters 3 and 4 of this thesis. Restriction digest was as follows: PCR products were precipitated in ethanol (Singh and Thomas, 2006) and re-suspended in 5 µL of nuclease free water. One µg of rDNA was digested with 10 units of the enzymes TaqI or Sau 96I, according to the manufacturer's (New England Biolabs) instructions and re-precipitated in ethanol. Pseudo TRFs formed at different stages of this procedure (Egert and Friedrich, 2003) were removed by digestion (10 min, 30°C) of DNA fragments with 0.5 units of mung bean

nuclease (New England Biolabs). The DNA was precipitated in ethanol, air dried in a fumehood, and re-suspended in capillary electrophoresis loading cocktail containing (μL): HiFi formamide 9, ultrapure water 1.425, ROX 400 size standard 0.075. These PCR and digestion reactions were repeated three times, and the fragments obtained were sized by capillary electrophoresis in a single run. The TRFs obtained were sized in an ABI 3130 gene sequencer using the Local Southern Method as the peak size-calling algorithm in the software GeneMapper v4.0 (Applied Biosystems, Foster City, CA). TRF fragment sizes are presented in Table 5.1.

5.3.4 Analysis of DSE and AMF in field root samples by terminal restriction fragment length polymorphism (TRFLP)

Root DNA was extracted according to the next procedure: 5 mg of ven dried (50°C , 24h) fine roots were ground in a bead miller. Two hundred μL of TE buffer and 50 μL of 20% Chelex 100 resin® in TE buffer were added to each tube, finger mixed during 20 seconds and heated at 95°C (5 min). The tubes were then incubated on crushed ice (1 min) and centrifuged at 10000 rpm (1 min). One μL of the supernatant was diluted in 199 μL of TE buffer and immediately used for PCR.

In each sample, DNA from AMF and DSE was amplified in replicate PCR reactions following a nested protocol. The first PCR reaction in a 20 μL volume contained 1 μL of diluted DNA, 18.34 μL of PCR platinum master mix HF (Invitrogen Corp) and 0.08 μL of a 50 μM solution of the eukaryotic specific primers LR1 [5'-3'] (GCATATCAATAAGCGGAGGA) and NDL 22 (TGGTCCGTGTTTCAAGACG) (Van Tuinen et al., 1998). The amplification included one step of 4 min at 94°C , followed by 35 cycles of 1 min at 93°C , 1 min at 60°C and 1 min at 68°C ; and a final step of 10 min at 68°C . The second PCR reaction with a labeled 5'FAM-LR1 and FLR2 [5'-3'] (GTCGTTTAAAGCCATTACGTC) primers (Van Tuinen et al., 1998) amplified the 5' end of the LSU rDNA of fungi in general. Amplification conditions were as

described above, except that 1 μ L of a 1/500 dilution of PCR product from the first reaction was used as a DNA template and the PCR amplification included 40 cycles. PCR products were always checked in agarose gels for the presence of about 600 and 800 bp DNA bands of DSE and AMF respectively. After the second reaction, PCR products from replicate reactions were pooled, and one μ L of DNA from this mixture was desalted and digested with 10 U of TaqI or Sau96I in parallel reactions. TRFs were finally digested with 0.5 U of mung bean nuclease. Enzymatic digestion and fragment sizing followed the procedures described above.

The TRFs present in more than one sample and in a range of ± 3 bases the length of theoretical TRFs of DSE and AMF (Table 5.1) were considered for analysis. The fragments were binned and aligned in the software T-REX (Culman et al., 2008), using one base as a clustering threshold, in agreement with the maximum length found to differentiate two phylogenetically distinct AMF or DSE (Table 5.1). One base difference in length between TRFs was also shown as the best value to recover the true composition of microbial communities in simulated TRFLP experiments (Abdo et al., 2006).

5.3.5 Soil temperature measurement

Soil temperature was recorded at 7.5 and 37.5 cm depth in the center of each plot at the time of root sampling. An “in house” built probe was used for its measurement. It consisted in a 45 cm long stainless steel rod, 9.5 mm in diameter, with a Type T (copper-constantan) thermocouple wire embedded in a narrow groove along the rod and sealed with epoxy resin. The temperature sensing junction was exposed on the surface, near the tip of the probe. Markings were stamped at 7.5 cm and 37.5 cm, and if needed, a hammer was used for deep soil insertion. Temperature values were captured on a Campbell Scientific 21X datalogger, and recorded two minutes after soil probe insertion in order to insure stable readings.

5.3.6 Data analysis

Aligned TRFs of AMF or DSE in root samples were analyzed as presence/absence values in T-REX, using an additive main effects and multiplicative interaction model (AMMI). AMMI has been demonstrated to have advantages over other ordination methods for analysis of TRFLP data (Culman et al., 2008). In the AMMI biplot estimated for the present TRFLP data, average values of DSE or AMF phylotype presence in a given plant species, as well as average phylotype presence across all plant species are presented along the X axis, while interaction scores between plant species and DSE or AMF phylotype presence are plotted along the interaction principal component axis (IPCA1) (Gauch, 2007; Zobel et al., 1988).

Since the AMMI analysis do not reveal which specific TRF was more frequent at a given plant species, sampling time or depth, AMMI analysis were followed by analysis of similarities (Anosim), a permutation based approach (Clarke, 1993) to compare overall AMF and DSE populations between seasons, plant species or its depths. In order to detect AMF or DSE phylotypes contributing more to the differentiation between populations, similarity percentage (Simpser) analysis was used when Anosim *P* values were significant (Clarke, 1993). Anosim and Simper were computed in the software Primer-e V5.

To further explore if there was a relationship between DSE and AMF community structures, distance matrixes computed from Bray Curtis similarity indices for each community were correlated by a Mantel test (Legendre and Legendre, 1998). A joint AMMI analysis of DSE and AMF communities was run in order to detect particular DSE and AMF contributing to this correlation. Partial mantel tests were used to further analyze the possible influence of soil temperature, plant species or soil horizon on the correlation between DSE and AMF community structures. Third matrixes were obtained from Euclidean distances between soil temperature values in each plot/depth, or built as design matrixes for plant species or soil horizons.

Table 5.1 Terminal restriction fragments (TRF) of AMF and DSE obtained from in vitro and computer simulated digestion of sequences or DNA

TRF length (bases)		Phylogenetic Classification ⁺	Genbank code	Plant host
In vitro	Simulated			
AMF cloned DNA digested with TaqI				
98.88 (0.66)*	103	<i>G intraradices</i>	EU380106	<i>P. virgatum</i>
192.08 (0.58)	191	GLO3	EU380084	<i>N. viridula</i>
235.09 (0.31)	232	GLO5	EU380126	<i>P. virgatum</i>
235.27 (0.31)	233	GLO5	EU380107	<i>P. virgatum</i>
236.19 (0.44)	234	GLO5	EU380083	<i>N. viridula</i>
237.20 (0.36)	235	GLO5	EU380085	<i>N. viridula</i>
238.31 (0.26)	236	GLO5	EU380032	<i>N. viridula</i>
546.97 (0.78)	557	GLO3	EU380086	<i>N. viridula</i>
DSE DNA digested with Sau96I				
38.10 (0.31)		AC4	--	<i>A. cristatum</i>
86.02		NV6	--	<i>N. viridula</i>
100.18 (0.39)	101	<i>Leptosphaeria</i> sp.	EU635773	<i>B. gracillis</i>
136.82 (0.37)		PV12	--	<i>P. virgatum</i>
136.84 (0.32)	135	<i>Phialophora</i> sp.	EU635769	<i>A. cristatum</i>
136.87 (0.32)		AC7	--	<i>A. cristatum</i>
137.31 (0.37)		PV9	--	<i>P. virgatum</i>
142.81 (0.32)		PS8	--	<i>P. smithii</i>
142.82 (0.22)	141	<i>Phaeosphaeria</i> sp.	EU635771	<i>P. juncea</i>
576.86 (0.16)		PJ5	--	<i>P. juncea</i>
597.0 (0.16)		AC15	--	<i>A. cristatum</i>
86.27 (0.38)		PS11	--	<i>P. smithii</i>
91.23 (0.37)		PV10	--	<i>P. virgatum</i>
91.33 (0.38)		PS14	--	<i>P. smithii</i>
99.68 (0.32)		AC3	--	<i>A. cristatum</i>

⁺ Based on analysis presented in Chapters 3 (DSE) and 4 (AMF).

* Values averaged from 3 digestions. In parenthesis the standard deviation.

--rDNA of these isolates was not cloned and only partial sequences are available upon request.

Procedures to build design matrixes were presented elsewhere (Legendre and Legendre, 1998). Mantel tests and Partial mantel tests statistics were obtained from 100000 randomizations in the software zt (Bonnet and Van de Peer, 2002)

5.4 Results

In all grasses studied, TRFLP analysis indicated changes in root DSE and AMF community composition during the growing season. Different phylotypes proliferated either, at different sampling times or in different soil horizons, although some phylotypes were also frequent in roots at all sampling times or soil depths (Figs. 5.1 and 5.2).

The AMMI biplot showed a changing pattern in DSE community composition as the growing season progressed (IPCA1 $P = 0.00000$). For instance, at both soil horizons, DSE phylotypes which were detected more frequently in May and August, were less frequent in October (Fig 5.1). These changes suggested an apparent gradual turnover of the DSE community as the growing season progressed, which was further supported by significant differences in DSE community composition in May and October (both sampling depths pooled, Anosim $P = 0.0001$). The overall DSE community composition in A and B horizons' roots of *N. viridula*, *A. cristatum* and *P. virgatum* was not significantly different (Fig. 5.1).

P. smithii was the only plant species which showed differences in DSE community composition between A and B horizons (Anosim $P < 0.05$). Simper analysis indicated that reduction in relative abundance of TRF 84 and 37, and increase in TRF 136 and 137 in October contributed more to the difference between DSE community composition in May and October (Fig. 5.1). The pattern of AMF distribution depended on plant species and soil horizons (IPCA1 $P = 0.0000187$), although it was different from that observed with DSE communities (Fig. 5.2). For instance, the community of AMF in roots of *N. viridula* did not change along the tree sampling times, while it was apparently changing from May to August in *P. smithii* (Fig. 5.2).

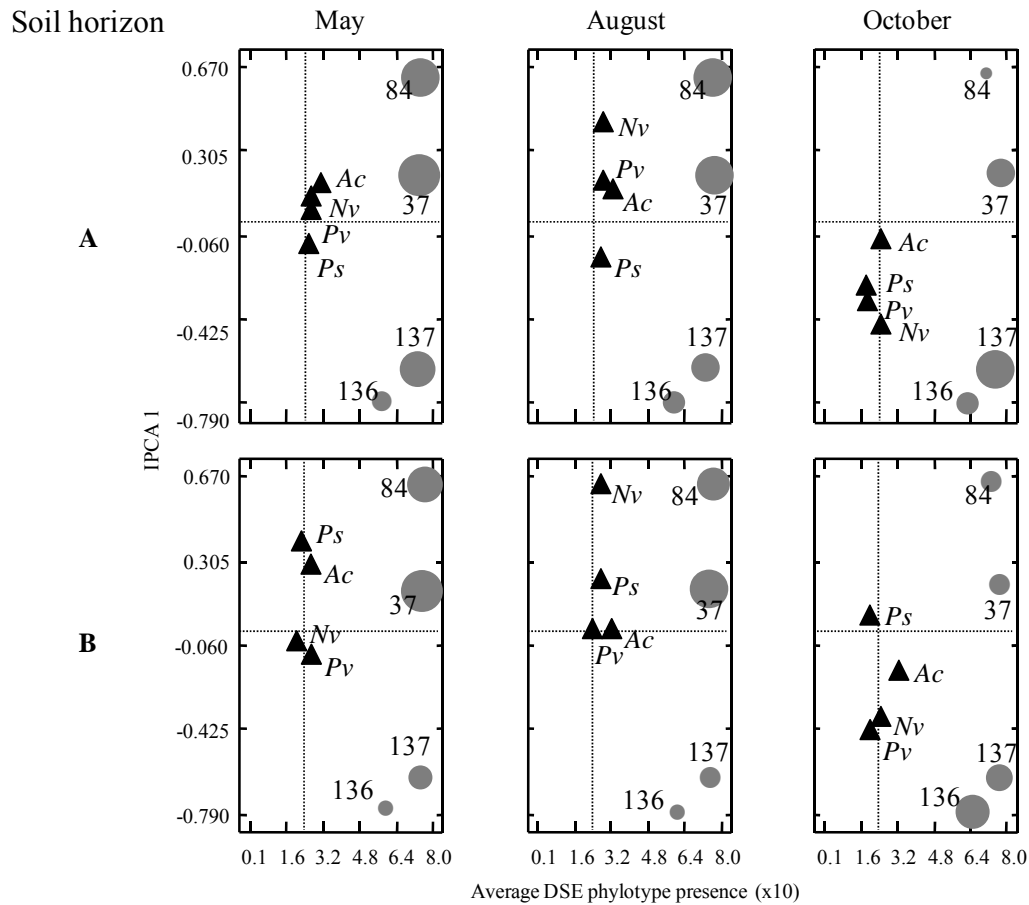


Fig. 5.1 Seasonal changes in DSE communities associated with roots of four prairie grasses in the A and B soil horizons. Triangles represent plant species and circles represent DSE phylotypes. Displacements of plant species along the vertical axis from one sampling time to another indicate changes in DSE composition. The size of the circles indicate relative abundance of DSE phylotypes at different sampling times (Simpser analysis). Only phylotypes with large contributions to differences in community composition are illustrated. Plant and fungal species with similar signs (+/-) along the IPCA axis indicate higher frequency of phylotype detection in that plant species. Codes for the grasses are Ac= *A. cristatum*, Nv = *N. viridula*, Pv = *P. virgatum*, Ps = *P. smithii*. Numbers beside circles are TRFs lengths which identify particular DSE isolates. The AMMI biplot was split by seasons and depths to aid in the visualization of changes in DSE community structure. Crossing dotted lines represent the centroid. This AMMI biplot accounts for 77.76% of the treatments sum of squares. N = 1209.

It was surprising that AMF was not detected in the roots of these two grass species in the B horizon in August, although they were detected at other sampling times. In August, TRFs 233 and 235 were the most frequent in roots of *P. virgatum* and *A. cristatum* (Fig. 5.2). The change in AMF communities in the B horizon between May and August was supported by Anosim ($P = 0.046$). Simper analysis indicated that the increase in relative abundances of TRFs 233 and 236 in August (Fig. 5.2) contributed to the difference in AMF communities in May and August (Fig. 5.2). Changes in overall AMF community composition in the A horizon at different sampling times or between depths at each sampling were not significant (Anosim $P > 0.05$).

The overall pattern of DSE and AMF detection in roots during the year suggest that the community of DSE in roots is more dynamic than that of AMF. The composition of DSE was different from May to October, while it was similar for AMF (Figs. 5.1 and 5.2), which showed a different composition only in August. This pattern of change also suggests that during the growing season, the interaction of DSE with roots may span a longer time period than for AMF.

The Mantel test further indicated a positive correlation between the structure of DSE and AMF communities (Table 5.2). Since the Mantel test was based on degrees of similarity, it indicates parallel changes in the complexity of DSE and AMF composition in the samples. For instance, the more complex was the community of AMF in a sample, more complex was the community of DSE in the same sample. Partial Mantel tests indicated that this correlation was independent from changes in soil temperature, depths or plant species (Table 5.3). AMMI analysis with both communities revealed the mutual exclusion of DSE and AMF communities, except for two DSE phylotypes that were more frequently detected near AMF phylotypes (Fig. 5.3).

5.5 Discussion

During the growing season root DSE and AMF composition changed. Some DSE and AMF

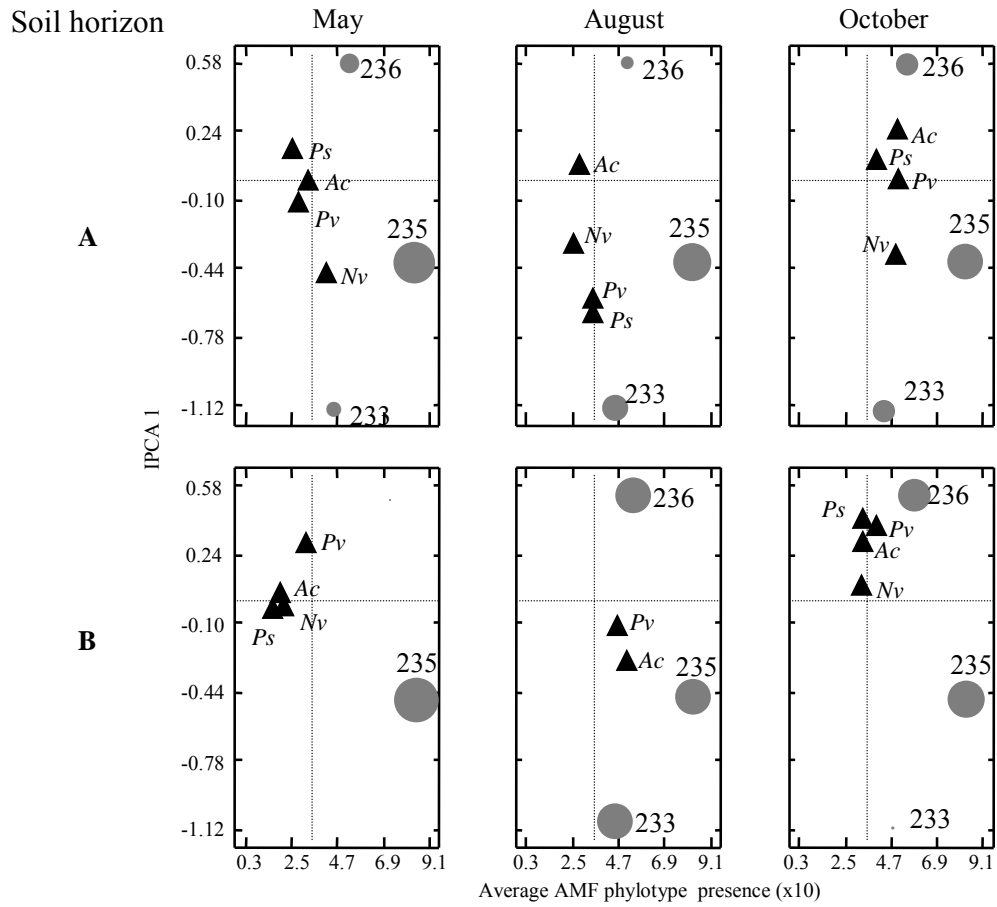


Fig. 5.2 Seasonal changes in AMF communities associated with roots of four prairie grasses at two soil horizons. Codes for the grasses and explanation of differences in circles size and numbers besides them is as in fig 5.1. This AMMI biplot accounts for 79.44% of the treatments sum of squares. N= 294

phylotypes proliferated at all sampling times and some other proliferated only at specific sampling times or in a specific soil horizon. Furthermore, DSE and AMF community structure was positively correlated.

The results of this research suggest the existence of multiple niches in prairie grass roots, which are separated in space and time. Different DSE and AMF populations appear to be differentially adapted to these multiple niches. These findings might provide clues about the relationships between environmental heterogeneity and root endophyte diversity, and perhaps about the joint role which DSE and AMF communities might play in plant processes in this ecosystem.

To understand the ecological value of the multipartite symbiosis between DSE, AMF and plants, it is necessary to consider the complex ecological structure that might develop in prairie grass roots as the season progress. In this environment, multiple niches for root endophytes might be created by the natural succession of environmental conditions, plant species and root phenologies. This creates a patchy distribution of niches even within the same piece of root, which are not static in time and that could be more efficiently exploited by plants engaged in transient symbioses with fungal endophytes differentially adapted to variable environmental conditions. Development of transient symbioses may allow symbiotic partners to use resources or tolerate stressful conditions (Schmidt et al., 2007) that could appear at different times during the growing season. Grassland ecosystems are characterized by large scale spatial and temporal fluctuations in temperature and resource availability, and it seems difficult to conceive single plants or single symbiosis successfully exploiting this scattered distribution of resources during a full growing season.

Root activity is dynamic and different niches should be always developing as root activity

Table 5.2 Correlation of AMF and DSE community structure with environmental factors at sampling. Mantel tests statistics (r_M , P) computed from 100000 permutations

Variable matrix	AMF		DSE	
	r_M	P	r_M	P
DSE	0.25	0.00514		
Soil Temperature	0.02	0.37094	-0.10	0.09819
Soil horizon	-0.01	0.49291	0.05	0.11195
Plant species	-0.03	0.11317	0.03	0.10309

Table 5.3 Influence of environmental factors on the correlation between DSE and AMF communities. Partial Mantel tests statistics (r_{PM} , P) computed from 100000 permutations.

Permutations conditioned by	Correlation DSE * AMF	
	r_{PM}	P
Soil Temperature	0.25	0.00437
Soil horizon	0.25	0.00447
Plant species	0.25	0.00494

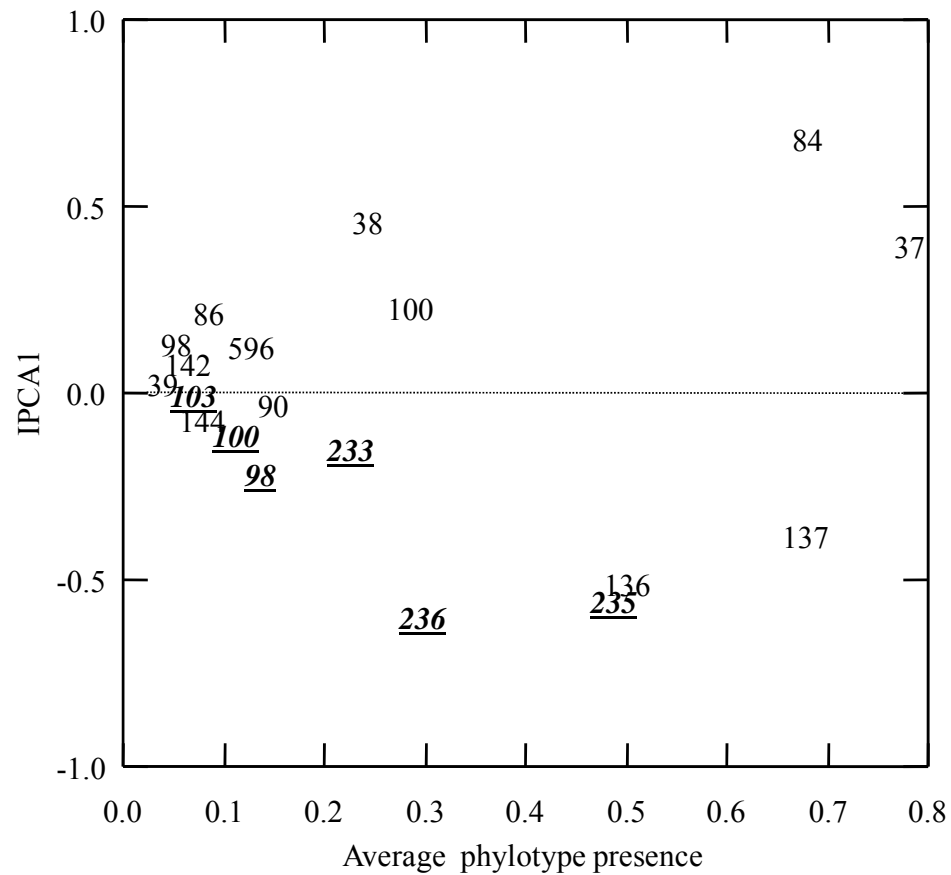


Fig. 5.3 AMMI analysis of DSE and AMF communities together. Numbers in bold and underlined represent TRFs length of AMF phylotypes. Un-bolled, not underlined numbers represent TRFs length of DSE. AMF and DSE TRFs with similar sign along the IPCA axis are positively correlated (N = 1503).

changes. High genetic diversity and differential adaptation in DSE and AMF to environmental conditions might make the tool which might promote the use of multiple niches with reduced competing interactions between DSE and AMF or between members of each fungal group.

The maintenance of high microbial diversity associated with plants is not easy to explain. Environmental heterogeneity appears to be important in maintaining such genetic diversity in root associated fungi (Koch, 2006). Transient symbioses might be used as the concept which harmonizes DSE and AMF diversity, plant development and ecosystem processes in the heterogeneous prairie environment. Some examples can illustrate this possibility. *Ranunculus adoneus* Gray is an alpine plant which showed high accumulation of N at the beginning of the growing season, concurrently with the highest level of root colonization by DSE and highest concentration of dissolved soil organic nitrogen. The highest accumulation of P in plant was found during the warmer part of the season, coincident with the peak in arbuscular colonization of roots by AMF fungi (Mullen and Schmidt, 1993). Similarly, the inoculation of the alpine herb *Gnaphalium norvegicum* L. with a DSE provided plant fitness by increasing germination at low temperature, while at higher temperatures AMF colonization increased plant N content and biomass accumulation (Ruotsalainen and Kytöviita, 2004). Such variable multipartite symbioses provide benefits for the plant at different times.

The DSE and AMF are genetically and physiologically distant organisms. AMF functioning appear to be more limited by low temperature than DSE functioning (Hetrick and Bloom, 1984; Kytöviita, 2005; Ruotsalainen and Kytöviita, 2004; Schmidt et al., 2007), and plant symbiosis with groups of organisms differentially adapted to variable environmental conditions might be advantageous in the prairie environment, which is characterized by drastic changes in soil temperature and seasonal availability of soil resources like water and nutrients. Under these

conditions, the development of transient symbioses should be an important strategy to extend plant use of resources.

Plant root occupancy is shared by DSE and AMF, and they are part of each other's environment. Complex interactions might occur among them. The correlation between DSE and AMF community structure might indicate symbiotic relationships between DSE and AMF phylotypes. But symbioses span from mutualistic to parasitic. Here DSE and AMF were mutually exclusive except for two DSE phylotypes which were positively related to the AMF community (Fig. 5.3). DSE fungi had been reported parasitizing hypha of root colonizing AMF (Mandyam and Jumpponen, 2008), and the isolation of different fungi with melanized hyphae from healthy AMF spores was reported before (Hijri et al., 2002; Verma et al., 1998). A DSE of the genus *Leptosphaeria* was isolated from healthy AMF spores (Hijri et al., 2002). It is interesting to note that two DSE isolates presented in Chapter 3 probably belong to the genus *Leptosphaeria* (Fig. 3.3) and at least one of them selectively promoted grass growth under reduced watering conditions. However, this TRF was detected with low frequency, and did not contribute to the differentiation between DSE communities at different sampling times. *Pyriiformospora indica*, a fungi with plant growth promotion capacity was also isolated from healthy spores of AMF (Verma et al., 1998).

One downside of the current molecular approach is that since DSE can grow inside AMF structures without apparent damage (Hijri et al., 2002) or in apparent parasitism (Mandyam and Jumpponen, 2008), DNA from both organisms would be amplified by PCR based methods independent on a parasitic or mutualistic relationship between them. However, because both organisms can play complementary roles, the consequences of any of these relationships for plant processes should not be the same. It is also possible that the restriction enzymes used might

have higher discrimination in particular AMF or DSE phylotypes, and other interactions were overlooked, or that the community of DSE in roots is under represented by a reduced number of isolates that grew up faster during the isolation procedure. These constraints should be considered in further studies. Nevertheless, the encounters of DSE and AMF in grass roots appear not to be casual. The reasons that allow the coexistence of these symbioses remain unknown, and deserve future attention.

In the grasses studied, DSE and AMF root colonization is highly dynamic, and this pattern of root colonization is different for each fungal community. Some members of each community dominate along the year, while some other proliferated at specific sampling times or soil depths. These transient symbioses with different DSE or AMF might provide differential plant access to soil resources or tolerance to variable stressful conditions along the year.

6 GENERAL DISCUSSION

The overall results of this research suggest that dark septate endophytic fungi (DSE) and arbuscular mycorrhizal fungi (AMF) adapt to specific environmental conditions as evidenced by DSE and AMF diversity shifts during the growing season and across soil horizons. Plant identity was also a driver of community composition. The symbioses involving multiple fungal phylotypes have spatio-temporal dimensions, suggesting that during the growing season these symbioses are functionally transient, rather than permanent phenomena. These findings are important contributions to knowledge and provide the basis to understand the nature and functioning of the DSE and AMF grass symbioses.

6.1 Harmonizing Spatio-Temporal Niche Dimensions With DSE and AMF Diversity

To understand the implications of grasses multipartite symbiosis with DSE and AMF in the prairie ecozone, it is necessary to consider the succession of environmental conditions, plant and root phenology as the growing season progress. At a finer scale, the change in climate and vegetation is accompanied by dramatic changes in root exudation within the same plant species (Henry et al., 2007) or in the type of sugars produced by C₃ and C₄ plants (Henn and Chapela, 2000) which dominate at different times. These changes might in turn affect fungal community composition or metabolism, and perhaps symbiotic performance on a temporary basis.

Niche distribution in prairie grass roots might appear to be extremely patchy, but it probably is not. Difficulties encountered in understanding plant symbioses with largely diverse DSE and AMF communities in the heterogeneous prairie environment, may be explained perhaps by the lack of an appropriate definition of niche, and of a relevant measure of diversity in DSE and AMF communities adapted to exploit them. As stated elsewhere, “Niche is not a place but an idea: a summary of the organisms tolerances and requirements” (Townsend et al., 2003; p 106).

From a fungal endophyte perspective roots are always changing, and some temporal plant and microbial stages might be more compatible with each organism tolerances and requirements. Although the definition of niche presented above might look too general, the challenge is to identify these niches in an always changing environment. It might in turn be useful in order to understand the ecological role of microbial root endophytes. Information from Chapters 4 and 5 illustrate this situation. It was found that there were depth specific assemblages of root AMF communities, which depended on the grass species analyzed (Fig. 4.4). This is important since it may indicate differential symbiotic plant access to deep soil resources. Depth differential composition in AMF was easily detected when different AMF phlotypes occurred at different soil depths. However, in roots of *P. virgatum*, one AMF phlotype, Glo5, apparently had different subpopulations colonizing roots at different depths (Fig. 4.4). This suggests that from an AMF perspective, the limits between different niches in the same plant might be well defined.

To support this discussion, I tested the hypothesis that subpopulations of this AMF phlotype are structured according to soil depths. The population of Glo5 was reanalyzed by computing values of genetic differentiation (Relethford, 2001) between subpopulations at different depths in each plant species. The results indicated that depending on the host plant, different subpopulations of Glo5 occurred at different soil depths (Fig 6.1). This genetic differentiation in sub populations of an AMF phlotype influenced by host plants is remarkable and might help to harmonize AMF genetic diversity with the environmental heterogeneity found in prairie grass roots. Values of genetic differentiation (F_{st}) between subpopulations of Glo5 at each soil depth in three of the grass species, and the absence of this phlotype in shallow root samples of *N. viridula* (Fig 6.1) suggest the existence of well differentiated niches in space.

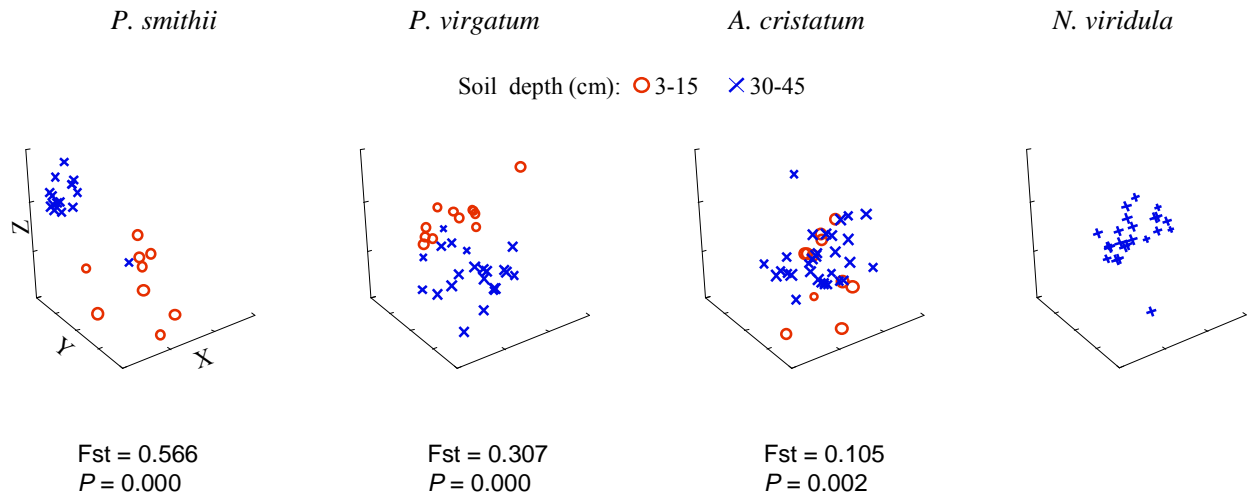


Fig. 6.1 Genetic differentiation in subpopulations of an AMF phylotype at two soil depths. Each symbol in a scatter plot representing a sequence of AMF rDNA of Glo5. The distance between symbols is proportional to the genetic distance between the sequences that they represent. Genetic distances, population differentiation (Fst) and its significance (*P* values obtained from 10100 permutations) were computed in the software Arlequin (Excoffier et al., 2005), and visualized by multidimensional scaling analysis (stress = 0.041).

This genetic differentiation also suggests extreme differences in the way in which different plant species appear to recruit and maintain levels of AMF diversity. For instance, in *A. cristatum* the F_{st} value indicated that only 10.5% of the total variation was explained by differences between subpopulations of Glo5 at each soil depth, and 89.5% was explained by genetic differences between ribotypes (rDNA sequences) within each subpopulation. This is in contrast with higher F_{st} values for Glo5 subpopulations in *P. smithii* (56.6%) and *P. virgatum* (30.7%) at different soil depths, suggesting greater separation among subpopulations influenced by soil depth, and lower differentiation between individuals composing these subpopulations.

It is also possible that instead of being different isolates of a same AMF species (i.e., subpopulations), Glo5 represents multiple AMF species, which share a common evolutionary history. The use of genes other than rDNA should provide clues about the origin of such genetic differentiation of Glo5, and the role of soil depth and plant species on this differentiation. This in turn will help to understand the maintenance of high genetic diversity and wide symbiotic performance of AMF.

It is interesting that Glo5 was the dominant AMF in the roots of *A. cristatum* and *P. virgatum* in deep, root samples from May and August of 2006 (Fig 4.4). In August of 2007, AMF were detected only in these two plant species in the B horizon, and some of the TRFs predominating at that specific time appear to be generated by Glo5 (Table 5.1). This suggests that niches for this particular phylotype are also separated in time, and supports the idea that AMF may adapt to very specific environmental conditions, including the phenology of the host plant. It is not known if this level of environmental adaptation may exist in DSE communities, although it might be possible. TRFs 136 and 137 appear to be generated by the same DSE phylotype, but do not behave similarly throughout the growing season in grass roots (Fig. 5.1) or in relationship

with the overall fungal community (Fig. 5.3). Furthermore, between isolate differences in functioning affected by plant phenological status and environmental conditions had been reported for *G. intraradices*, an AMF (Koch et al., 2004; Munkvold et al., 2004) and *Phialocephala fortinii* (Bartholdy et al., 2001), a DSE.

From these results, it is apparent that in prairie grasses, attempting to relate genetic diversity and ecosystem processes might require units of diversity defined at different taxonomical levels, and the consideration of niche spatio temporal dimensions.

6.2 Transient Symbiosis in Prairie Grass Roots

Plant symbiosis with organisms that are differentially adapted to environmental conditions might be advantageous in the prairie environment, which is characterized by drastic changes in soil temperature, vegetation, water and nutrient availability (Schmidt et al., 2007). Under these conditions, the development of transient symbioses might be an important strategy to extend plant use of resources. It is difficult to conceive a single symbiosis successfully exploiting the scattered distribution of resources as they appear in prairie environments. It had been shown before that the temporal separation in a multipartite symbiosis provides benefits to plants at different times (Mullen and Schmidt, 1993; Ruotsalainen and Kytöviita, 2004), which may explain the investment of organic carbon into different specialized endophytes. DSE and AMF are genetically and physiologically distant organisms, and they appear to have different and contrasting environmental limits for functioning. AMF activity appears to be limited by low soil temperature (Hetrick and Bloom, 1984; Ruotsalainen and Kytöviita, 2004) while DSE can show high metabolic activity or proliferate in roots in these conditions (Kytöviita, 2005; Schmidt et al., 2007). It is also important to realize that differences in environmental adaptation and functioning at the subspecies level were shown in both DSE and AMF (Bartholdy et al., 2001; Koch, 2006; Munkvold et al., 2004; Queloz et al., 2005; Sieber and Grünig, 2005).

The genetic diversity of AMF found in this research could be largely influenced by the host plant, but plants are only a part of the environment for these fungi, at least in the case of AMF, which are more soil fungi connecting to roots rather than root inhabitant; the growth form of DSE in soil is largely unknown. In addition, the root environment is complex, and DSE and AMF are also part of each other's environment. Although DSE and AMF show different levels of biotrophy, in symbiosis both can have access to plant synthesized organic compounds (Usuki and Narisawa, 2007). Data in Fig. 5.3 suggest a partial separation of DSE and AMF communities, implying that different stages of symbiosis, from mutualism to parasitism might occur between DSE and AMF. It is not known whether DSE and AMF compete for supplied plant C, but it was suggested that competing interactions for common resources between root associated fungi might lead to a separation in functionally and physiologically differentiated communities (Gadgil and Gadgil, 1971).

From this perspective, the presence and activity of DSE or AMF in creating a heterogeneous environment for each other is as important as the successional changes in climate and vegetation during the growing season. It is necessary to discover how DSE and AMF interact in plant roots and the consequences of this interaction on plant processes. DSE have been found parasitizing (Mandyam and Jumpponen, 2008) or inhabiting inner AMF structures without apparent damage (Hijri et al., 2002). Furthermore, a fungi with capacity to promote plant growth and affect genetic expression of cell roots (Waller et al., 2005) was isolated from healthy spores of AMF (Verma et al., 1998).

Under water stress, DSE were involved in the carbon economy of grasses and one isolate increased plant N concentration (Chapter 3). AMF had also been involved with plant N nutrition (Govindarajulu et al., 2005) and water relations (Augé, 2001). Since water availability and N are

the most limiting growth factors in the prairie ecozone, it seems possible that the most successful plants in this environment are those engaged in a higher number of transient symbiosis, while keeping a positive plant carbon balance. Future research might turn this possibility into experimental facts.

6.3 Future Research

The multipartite symbiosis with dark septate endophytes and arbuscular mycorrhizal fungi appear to be widely distributed in many plant species common to the prairie ecozone.

Parallel changes in climate, vegetation and soil resource availability create highly heterogeneous conditions to which DSE and AMF populations appear to be differentially adapted. Future research efforts should extend the knowledge gained from this research regarding diversity, distribution and functions of these fungal communities, as well as the mechanisms which allow their coexistence in plant roots.

Communities of DSE and AMF appear to be composed of genetically complex lineages that are differentially distributed in spatial-temporal structures. Future assessments of diversity might be greatly benefited if these dimensions are considered. For instance, the collection of DSE isolates was obtained from a single sampling period, and it seems possible that more isolates may be obtained at different times of the year and at different soil depths. In assessments of community biodiversity and dynamics, it may be also interesting to narrow the scales of sampling times within a single season, and to know whether the annual patterns of DSE and AMF distribution are repeated year after year in a given point in time. If that is the case, it will be possible to model the development of DSE and AMF communities during the growing season, along with any well identified process influenced by them.

Nitrogen and soil water availability are the most limiting conditions for plant growth in the prairies (Willms and Jefferson, 1993). DSE and AMF can be involved in plant-soil relations; the

local fungal isolates examined in my research seem to be involved in this type of relationship. The extent of such involvement in interacting DSE and AMF communities varying with plant phenology and photosynthetic system might be also a topic of interest.

However, a prerequisite to gain more insight about the involvement of fungal endophytes in plant and ecosystem processes will require a better understanding of the taxonomic relationships among individuals composing these communities. The continued use of terms like DSE or AMF may not provide a clear path to understand these relationships. Nonetheless, genetic features of fungal species and communities associated with environmental processes need to be identified, as well as the environmental conditions which shape their short term evolution. Prairie grasses appear to offer unique opportunities to gain more insight in these fundamental aspects of biology.

7 LITERATURE CITED

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APPENDIX

EVALUATION OF PRIMER PAIRS FOR THE ANALYSIS OF FUNGAL ROOT ENDOPHYTIC BIODIVERSITY

Note: The following text has been reformatted here and was previously published as:

Perez, J., C. Hamel, J.J. Germida, and M.P. Schellenberg. 2009. Evaluation of primer pairs for the analysis of fungal root endophytic biodiversity, p. 1255-1263, *In* A. Chauhan and A. Varma, eds. A textbook of molecular biotechnology. I.K. International Publishing House Pvt Limited, New Delhi.

Learning Objectives

Acquire the knowledge and techniques for the evaluation of suitable primers sets used in molecular assessments of fungal root biodiversity.

Introduction

The diversity of fungi that can develop symbioses with plant roots is remarkable. Fungal root endophytes are referred to as arbuscular mycorrhizal fungi (AMF) or dark septate endophytes (DSE). AMF form a taxonomic group, the Glomeromycota, and many fungal root endophytes that do not belong to this phylum are placed in an artificial group called DSE. Under specific conditions these associations can provide nutritional benefits and stress tolerance to plants (Lekberg and Koide, 2005; Mandyam and Jumpponen, 2005). Both AMF and DSE can colonize the same plant species in different ecosystems (Mandyam and Jumpponen, 2008; Mullen et al., 1998).

Research on fungal root endophytes is limited by their high genetic diversity and the difficulty to grow many of them on culture media (Berch et al., 2002). Molecular methods are very useful to study the diversity of fungal endophytes in roots. By amplifying with the polymerase chain reaction (PCR) fragments of DNA in regions that are conserved within the Kingdom Fungi,

specific DNA sequences can be obtained. Information on the identity of the organisms possessing these sequences is then sought by comparison to sequences of known organisms available in data bases and by phylogenetic analyses. Examples of these techniques have been presented in research with root endophytes (Gollotte et al., 2004; Renker et al., 2006; Schadt et al., 2001).

The choice of PCR primer pairs is critical in biodiversity studies based on the analyses of DNA sequences. Primers need to be specific enough for DNA amplification only from members of the target group of organisms and general enough to amplify the DNA of most, if not all, members of that particular group. The specificity and range of PCR primers should first be tested on reference microorganisms. Hagn et al., (2003), provided a means to test the specificity and range of amplification of fungal primers sets, and demonstrated that primer pairs should be tested using reference fungal cultures rather than the mere genetic information found in data bases.

The first primers intended to amplify DNA from AMF were designed by (Simon et al., 1992). Numerous primers targeting different portions of ribosomal DNA (rDNA) genes were developed since then to study fungal root endophytes or soilborne fungi (Clapp et al., 1995; Vandenkoornhuyse et al., 2002). Examination of the scientific literature on the topic reveals that some of these primers are widely used. However evidence of the narrowness of their range or their lack of specificity has also been reported (Schüßler et al., 2001).

The adequate amplification of DNA from DSE and AMF by previously published primer pairs was tested. The objective of this chapter is to communicate how I selected primer pairs appropriate for the study of fungal root endophyte biodiversity at one experimental site near

Swift Current SK (latitude: 50.17N, longitude: 107.41W, altitude: 818m) in the Canadian prairie ecozone.

Biological Materials

DNA of DSE and AMF from culture collections and from field samples, and that of their host plants (Table A.1) were used to test the suitability of previously published primer pairs. Fifteen DSE were isolated from surface sterilized roots of field-grown prairie grasses according to the following procedure: Roots were sterilized by immersion in 95% ethanol for 10 sec, in sterile water for 10 sec, in 2.5% Javex® (sodium hypochlorite) for 2 min, and in sterile water for 2 min. Some 0.5 cm long pieces of root were cut with a sterile scalpel and plated on PDA supplemented with neomycin sulfate and streptomycin sulfate (Vujanovic et al., 2002) and incubated in the dark at 25°C. Hyphae emerging from the cut ends were transferred to new Petri plates to obtain pure cultures.

Roots of field grown crested wheatgrass plants (*Agropyron cristatum* L) were microdissected to separate chlamidospores of two unknown fungi and extract their DNA. DNA extracted from the roots of leek (*Allium porrum* L.) trap cultures of AMF from different grass stands were also used in the assessment of primer suitability. The DNA from leek leaves served as negative control in the tests (Table A1).

DNA Extraction and PCR Amplification by Popular Primer Pairs

DNA from lyophilized plant and fungal cultures was extracted with the DNeasy plant mini kit (QIAGEN Inc.). DNA from AMF spores and roots containing chlamydo spores was extracted by crushing 20 spores in 10 µL of TE buffer and pipetting the suspension into bead tubes contained in the Ultraclean Soil DNA extraction kit (MoBio laboratories Inc). The instructions of the manufacturer were followed for each system.

The primers evaluated in this study are listed in Table A.2. EF3 and EF4 is a primer pair initially developed to amplify the short subunit (SSU) of rDNA gene's of fungi in wheat rhizosphere (Smit et al., 1999). These primers had been shown to be fungi specific in former tests (Hagn et al., 2003), although the priming sites were not present in some AMF (Smit et al., 1999). LR1- FLR2 is also known as a primer pair for general fungi, but targets the long subunit (LSU) of the rDNA gene (Van Tuinen et al., 1998). Primers AMV 4.5NF-AMDGR (Sato et al., 2005), 28G1-28G2 (da Silva et al., 2006), and LR1-FLR4 (Gollotte et al., 2004) are claimed to specifically amplify DNA from AMF. The last two primer pairs target the LSU rDNA gene and were also used in a nested PCR after amplification with LR1-FLR2 (Table A.2).

For PCR amplification, 10 µL of PCR reaction mixture was prepared using AmpliTaq Gold PCR master mix (Applied Biosystems). The final concentration of each component of the reaction mixture is as follows: 15mM Tris HCL/pH 8.5; 50 mM KCL; 200 µM each dNTP, 2.5 mM MgCl₂, 0.25 enzyme units of Amplitaq gold DNA polymerase; 0.5 µM each primer. 1 µL of crude DNA was used as a template, except in nested PCR reactions, where 1 µL of a 1/500 dilution of the product of the first PCR reaction was used in the second PCR reaction. PCR reactions were conducted under the conditions specified by the authors listed in Table A2, except that an initial step of 10 min at 95°C was included to activate the enzyme. Each reaction was repeated three times, and PCR products were analyzed by electrophoresis in agarose gels.

Most of the DNA from DSE was amplified by both, the LR1-FLR2 and EF3-EF4 primer pairs, although DNA bands from micro-dissected roots were produced only with LR1-FLR2 (Table A.3). Amplifications with the primer pair AMV4.5NF–AMDGR were not specific to AMF. A band was produced from the DNA of leek leaves, indicating amplification of plant

DNA with this primer pair. The same band was present in the amplification products of colonized leek root DNA extracts (Fig. A.1). This primer pair was not evaluated in further tests.

Specific amplification of AMF sequences was produced by primers LR1-FLR4 and 28G1-28G2. The AMF sequence detection power of these primer pairs was improved with the use of a nested PCR protocol using the general fungi primer pair LR1-FLR2, in a first round of amplification (Table A.4).

Further Evaluation of Primers by Cloning and Sequencing of PCR Products

The performance of the best primer pairs, as shown in the previous test, were evaluated based on the cloning and sequencing of PCR products. DNA obtained from DSE cultures was amplified with the primer pair LR1-FLR2. A nested protocol using the primer pair LR1-FLR4 after a first amplification with LR1-FLR2 was applied to AMF DNA from extracts of colonized leek roots and field-grown crested wheatgrass roots. PCR products were cloned into the pCR4 topo vector and transformed into TOP10 chemically competent cells of *Escherichia coli* following the instructions of the manufacturer (Invitrogen Corp.). Positive transformants containing AMF or DSE nucleotide sequences were screened by direct PCR amplification of 1 uL of bacterial culture, with primers LR1-FLR4 or LR1-FLR2. Sequencing of AMF rDNA fragments contained in 38 bacterial clones and DSE rDNA fragments in five bacterial clones were commercially sequenced using standard vector targeting primers, at the Plant Biotechnology Institute (National Research Council, Saskatoon, SK, Canada).

The nucleotide sequences obtained in this study were aligned in Clustal W (Gibson et al., 1994) and a phylogenetic tree was built using the Neighbor-Joining method (Saitou and Nei, 1987).

Table A.1. Sources of DNA used in primer pair suitability assessment

Sample	Source
AMF spores	
<i>Glomus intraradices</i> DAOM181602	GINCO CAN
<i>Archaeospora trappei</i> AZ119A	INVAM
<i>Paraglomus occultum</i> MT900	INVAM
<i>Scutellospora callospora</i> NY340	INVAM
Roots leek trap cultures containing	Study site
<i>Glomus claroideum</i> DAOM 235379	Study site
AMF from crested wheatgrass (<i>A. cristatum</i>) stands	Study site
AMF from western wheatgrass (<i>P. smithii</i>) stands	Study site
Leek leaves	n/a
Colonized roots of field-grown crested wheatgrass	Study site
DSE pure cultures	
Isolates #1 to 14 and #17	Study site
Chlamydospores from crested wheatgrass root microdissection	
Isolates #15 and 16	Study site

Table A.2 Primers assessed in this study

Primer name	Sequence (5' 3')	Target organism	Reference
LR1	GCATATCAATAAGCGGAGGA	Eukaryotic	(Van Tuinen et al., 1998)
FLR2	GTCGTTTAAAGCCATTACGTC	General Fungi	(Trouvelot et al., 1999)
EF3	TCCTCTAAATGACCAAGTTTG	General fungi	Smit et al., 1999)
EF4	GGAAGGG[G/A]TGTATTTATTAG	General fungi	Smit et al., 1999)
FLR4	TACGTCAACATCCTTAACGAA	AMF	(Gollotte et al., 2004)
28G1	CATGGAGGGTGAGAATCCCG	AMF	(da Silva et al., 2006)
28G2	CCATTACGTCAACATCCTTAACG	AMF	(da Silva et al., 2006)
AMV4.5NF	AAGCTCGTAGTTGAATTTCG	AMF	(Sato et al., 2005)
AMDGR	CCCAACTATCCCTATTAATCAT	AMF	(Sato et al., 2005)

The bootstrap consensus tree was inferred from 10000 iterations (Felsenstein, 1985) and branches reproduced in less than 50% bootstrap replicates were collapsed. Evolutionary distances based on substitutions per position were computed using the Tajima-Nei method (Tajima and Nei, 1984). All positions containing gaps and missing data were eliminated from the dataset and 491 positions were included in the analyses. Phylogenetic analyses and tree drawing were conducted in MEGA4 (Tamura et al., 2007). Six sequences of AMF (*Glomus* sp. AJ854647.1, *G. mosseae* AJ628051, *G. mosseae* DQ469128.1, *Glomus* sp. AM040432, *G. intraradices* AY373433, *G. occultum* AFTOL) and 4 from DSE (*Cryptococcus victoriae* AM160647.1, *Phialophora lagerbergii* AB10622, *Phaeosphaeria* sp. EF590319.2, *P. kuwaitiensis* AJ849362.1) giving the closest match in a Blast (Wheeler, 2003) search with the sequences obtained, were included in this analysis

Discussion

The sequences obtained with the AMF-specific primers LR1-FLR4 yielded different degrees of similarity with AMF sequences contained in Genbank. Best hits of sequences obtained were found with sequences of *Glomus intraradices* or *G. mosseae*. Sequences obtained from pure cultures of DSE using the primer pair for general fungi LR1-FLR2 resulted in good matches with members of the Ascomycota and Basidiomycota, two phyla known to include DSE or ectomycorrhizal forming fungi (Table A5) and no ambiguous matches with organisms different from fungi were found.

The phylogenetic analyses classified AMF and DSE in clearly distinct groups. Sequences giving weak similarity values with known fungi were appropriately classified in their respective groups of AMF or DSE (Fig. A.2). This strongly supports the specificity of LR1-FLR2 towards

fungi in general and of LR1-FLR4 towards mycorrhizal fungi. The identification based on Blast searches should be complemented with phylogenetic analyses, because databases can contain errors or be incomplete (Jumpponen, 2007; Schüßler et al., 2003).

It is important to note that the performance of the primer FLR4 is greatly improved by a nested protocol using LR1-FLR2 in a first PCR reaction. It is probable that part of the mismatches for FLR4 are eliminated by primer FLR2 during the first PCR cycle, since there is an overlapping portion in the primers. On the other hand, more specificity is also granted to FLR2 by the use of clean DNA samples, such as those obtained from pure cultures of DSE isolates. It is recommended to use this primer in a nested PCR protocol with the eukaryotic primer NDL22 (Van Tuinen et al., 1998) when used with environmental samples.

Insuring the specificity of the primer pairs used is important in biodiversity studies where conclusions are drawn from unidentified tagged DNA fragments or bands, such as those produced by Terminal Restriction Fragment Length Polymorphism (TRFLP) or Denaturing Gradient Gel Electrophoresis (DGGE), two popular environmental DNA analysis methods. The risk of non specific amplification of microbial operational taxonomic units using these PCR based strategies is high. For example, Ma et al., (2005) in a study on AMF biodiversity in soil extracted bands of DNA from a DGGE gel after amplification with primers AM1–NS31. Sequencing and sequence comparison with a Blast search results revealed that the sequence found shared 99% similarity with *Phialophora verrucosa*. Clearly these primers are not AMF-specific and may lead to erroneous conclusions if the amplified sequences are not duly identified. It is also important to consider that even the DNA extracted from a single spore of an AMF, can contain a mixture of DNA from different organisms.

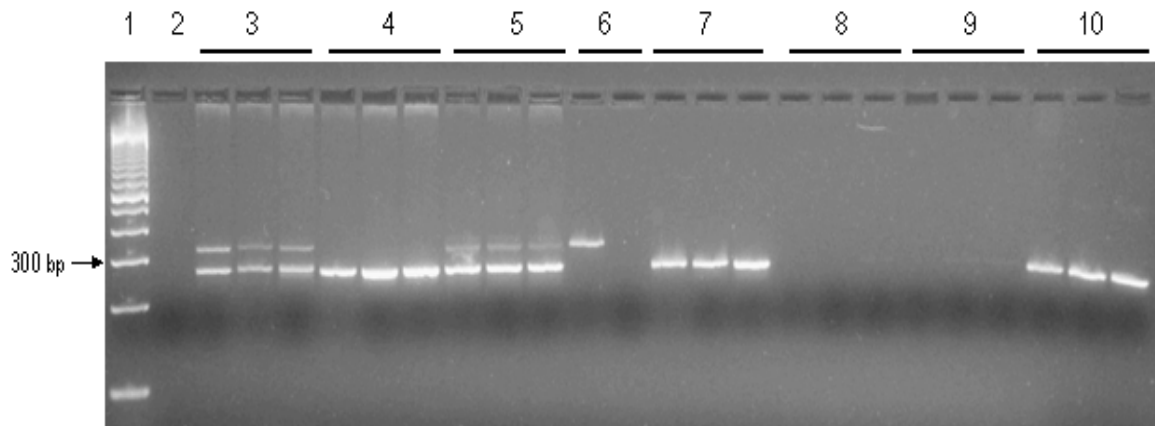


Fig A.1 Non AMF-specific amplification of DNA by primers AMV4.5NF-AMDGR. Lines : 1, 100 bp marker; 2, Blank; 3 to 5, roots from leek trap cultures of AMF from crested wheatgrass stand, from western wheatgrass stand, and a pot culture of *G .claroideum*, respectively; 6, DNA from leek leaves; 7 to 10 DNA from *G. intraradices*, *A. trappei*, *.P. occultum* and *S. heterogama*, respectively.

Table A.3. Amplification of DNA from DSE isolates #1 to 17 by two primer pairs.

Primer pair	DSE isolate number																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
LR1-FLR2	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
EF3-EF4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+

+ DNA band was present in agarose gel
- Band was absent

Table A.4 Specific amplification of DNA from arbuscular mycorrhizal fungi (AMF) by two primer pairs used in a single or in a nested PCR protocol.

Primer pair	<i>G. claroideum</i>	<i>G. intraradices</i>	<i>P. occultum</i>	<i>S. heterogama</i>
Single PCR				
LR1-FLR4	+	+	-	-
28G1-28G2	+	+	+	+
Nested PCR after LR1-FLR2				
LR1-FLR4	+	+	+	+
28G1-28G2	+	+	+	+
+ DNA band was present in agrose gel				
- Band was absent				

Table A.5 Similarity between clones obtained in this study with LSU rDNA from known fungi deposited in Genbank.

Entry and accession number in Genbank	Clones	Maximum similarity (%)
AMF		
<i>Glomus</i> sp AJ854592.1	3	96-97
<i>Glomus</i> sp AM040435.1	10	92
<i>Glomus</i> sp AJ854625.1	3	98-99
<i>Glomus</i> sp AJ854619.1	1	99
<i>Glomus</i> sp AJ854615.1	1	98
<i>Glomus</i> sp AJ854647.1	4	98-99
<i>G.deserticola</i> AJ746249.1	1	88
<i>G.intraradices</i> AY373433.1	6	98
<i>G.intraradices</i> AJ854575.1	1	97
<i>Glomus</i> sp AJ854626.1	1	98
<i>G. diaphanum</i> DQ469113.1	1	86
<i>G. mosseae</i> DQ469128.1	6	99
Total clones AMF	38	
DSE		
<i>Phialophora lagerbergii</i> AB100622.1	1	99
<i>Leptosphaeria calvescens</i> AY849944.1	2	96
<i>Phaeosphaeria</i> sp EF590319.1	1	97
<i>Massarina ignaria</i> DQ810223.1	1	99
Total clones DSE	5	

Concluding remarks

For PCR based analyses of fungal root symbionts, it is necessary to test the specificity of primers using reference microorganisms and local isolates. It was found that the primer pair LR1-FLR2 is very specific towards fungi in general, and LR1-FLR4 in a nested PCR with LR1-FLR2 is very specific for AMF, at least in samples from this study site.

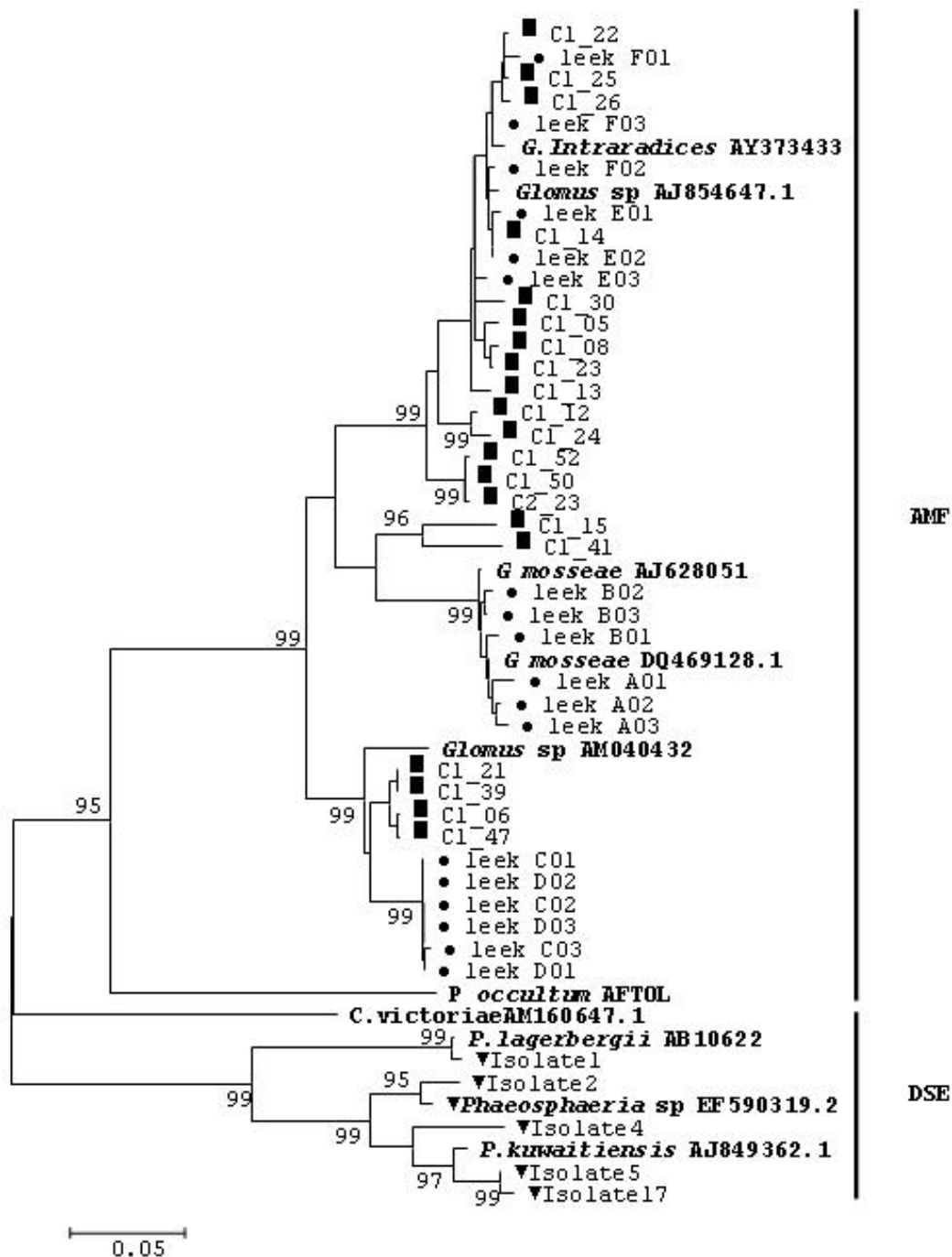


Fig. A.2 Phylogenetic analysis of fungal LSU rDNA gene sequences obtained in this study or downloaded from Genbank. Values next to the branches represent the percentage of replicate trees in which the associated clones clustered together in the bootstrap test (10000 iterations). ■ DNA sequences from roots of field grown crested wheatgrass; ● DNA sequences from the roots of various leek trap cultures of AMF; ▼ DNA sequences of DSE. In bold sequences obtained from Genbank.